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ABSTRACT

Oxidative stress plays an important role in the pathogenesis of cardiovascular diseases. It may cause the modification and activation of proteins such as matrix metalloproteinases (MMPs) and AMP-dependant protein kinase (AMPK) which are implicated in the development of cardiac diseases. I investigated their role in the development of cardiac contractile dysfunction caused by oxidative stress in two different models: a) the direct infusion of reactive oxygen species into the heart and, b) endogenous oxidative stress caused by ischemia and reperfusion (I/R) injury.

In isolated working rat hearts exposed to a bolus of hydrogen peroxide (H_2O_2), I found that AMPK and MMP-2 were activated in parallel with the contractile dysfunction caused by H_2O_2. The use of pyruvate as an antioxidant prevented both AMPK activation and release of MMP-2. However, cardiac dysfunction was prevented by pyruvate but not by MMP inhibitors. In contrast, isolated cardiac myocytes subjected to a continuous infusion of peroxynitrite ($ONOO^-$) developed contractile dysfunction along with activation of MMP-2. The MMP inhibitors doxycycline or PD 166793 prevented contractile dysfunction in this model. I also attempted to determine whether MMPs inhibition by doxycycline reduces contractile dysfunction in myocytes challenged with a single bolus of $ONOO^-$ by inhibiting the proteolysis of sarcomeric proteins by MMPs. Interestingly, I found that $ONOO^-$ almost enhances ($p=0.06$) the degradation of myosin light chain 1 (MLC1) as well as doxycycline prevented the reduction in cell viability caused by $ONOO^-$. Doxycycline not only inhibited MMPs but also partially scavenged $ONOO^-$.

Our lab has previously shown that endogenous oxidative stress produced by I/R in the heart activates MMP-2 which then proteolyzes specific intracellular protein targets such as troponin I. Therefore, I examined in isolated rat hearts subjected to I/R whether MMP-2 can degrade other proteins besides troponin I using a pharmaco-proteomics approach. I found that MMP-2 co-localizes with MLC1 in the thick filament of cardiac myocytes. In addition, MMP-2 degrades MLC1 in cardiac I/R which is prevented by MMP inhibitors. I found that the actual cleavage site in which MLC1 is degraded by MMP-2 is located between tyrosine 189 and glutamine 190 at the C-terminal domain.

In summary, these studies show preventing oxidative stress or inhibiting the activation of MMPs in the heart holds promise in the treatment of cardiac diseases.

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Dedicated to my parents

and

my lovely wife Juliana

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Abbreviations

^{o}C: degrees celsius

<: less than

>: more than

=: equal

±: plus or minus

g: g force

µg: microgram

mg: miligram

ACC: acetyl-CoA carboxylase

ADP: Adenosin diphosphate

AMP: Adenosin monophosphate

ATP: Adenosin triphosphate

AMPK: AMP-dependant protein kinase

AMPKK: AMPK kinase

Ca^{2+}: Calcium

[Ca^{2+}]: Calcium concentration

CMT: chemically modified tetracyclines

CoA: coenzyme A

Cu/ZnSOD: cupper/zinc superoxide dismutase

eNOS: endothelial nitric oxide synthase

FAD

FADH

GSH: glutathione

H^+: hydrogen ion

H_2O_2: hydrogen peroxide

HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A

HOCl: hypochlorous acid

iNOS: inducible nitric oxide synthase

IEF: isoelectrical focusing

IPG: immobilized pH gradient

I/R: ischemia-reperfusion

IU: international units

MAO: Monoamine oxidase

MLC1: myosin light chain 1

MMPs: matrix metalloproteinases

MnSOD: manganese superoxide dismutase

MT-MMP: membrane type MMP

$NO^{\bullet}$: nitric oxide

NOS: nitric oxide synthases

nNOS: neuronal nitric oxide synthase

$O_2^{\bullet -}$: superoxide anion

$OH^{\bullet}$: hydroxyl radical

ONOO$^-$: peroxynitrite

ONOOH: peroxynitrous acid

PDH: pyruvate dehydrogenase

ROS: reactive oxygen species

SH: sulfhydryl

SOD: superoxide dismutase

XOR: xanthine oxido-reductase

CHAPTER 1

INTRODUCTION

1.1. OXIDATIVE STRESS IN THE HEART

Cardiovascular disease is the leading cause of morbidity and mortality in the world.[1] During the last two decades a striking amount of research has been done in order to elucidate the molecular basis of cardiovascular disease not only to understand the related pathophysiological mechanisms but also with the purpose of finding some novel pharmacological approaches for its treatment. It has been reported that several cardiovascular diseases are characterized by an increase in oxidative stress resulting in increased production of highly reactive molecules such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), hypochlorous acid (HOCl), lipid radicals, nitric oxide ($NO^{\bullet}$) and peroxynitrite ($ONOO^{-}$) which outstrip the organism's endogenous antioxidant defence.[2] Oxidative stress has been implicated in a number of processes such as oxidation of biological macromolecules such as DNA, carbohydrates and lipids as well as different post-translational modifications of proteins including nitration, nitrosylation glutathiolation and oxidation.[2-4] Oxidative stress has been observed in many different pathological settings such as cancer, diabetes, sepsis and cardiovascular diseases. This section will be mainly focused in the role of oxidative stress in heart disease.

The enhanced production of reactive oxygen species (ROS) was initially observed in the context of cardiovascular diseases in the 1970s. Naito showed by electron spin resonance the generation of free radicals in myocardial infarction in dogs.[5] In addition, Lankin *et al*,[6] showed the formation of lipid peroxides in atherosclerosis. Recently, Ide *et al*,[7] demonstrated in dogs with pacing induced heart failure an elevation in the rate of electron spin resonance decay proportional to $OH^{\bullet}$ level that was correlated with the compromise in left ventricular function. Furthermore, it has been observed that direct administration of $ONOO^-$ into the heart decreases cardiac efficiency independently from changes in myocardial oxygen consumption.[8] In addition, increased levels of $ONOO^-$ can be observed during pro-inflammatory cytokine-induced heart failure (resembling that seen during septic shock) as a consequence of the enhanced production of both $O_2^{\bullet-}$ and $NO^{\bullet}$ in the heart.[9] Ischemia and reperfusion results in increased endogenous oxidative stress. It was reported that $ONOO^-$ plays an important role in the cardiac dysfunction present during the reperfusion period.[10,11] The deleterious effects of $ONOO^-$ were inhibited by exogenous administration of glutathione (GSH), a natural antioxidant which scavenges $ONOO^-$.[12] It is important to clarify that in ischemia-reperfusion injury not only $NO^{\bullet}$, $O_2^{\bullet-}$ and $ONOO^-$ are produced but also other ROS. Crestanello *et al*[13] observed in isolated rat hearts using chemiluminiscence that H_2O_2 is also present and causes cardiac dysfunction during reperfusion that is blunted with the administration of pyruvate, a scavenger of H_2O_2 and $OH^{\bullet}$, in addition to its role as a substrate in carbohydrate metabolism.

The scope of this chapter is to review the role of oxidative stress in the heart, the origin and sources of ROS as well as mechanisms by which the cardiovascular system protects itself from oxidative stress. In addition, this section will describe the pathogenesis of myocardial stunning during ischemia and reperfusion injury and review the roles of two different protein targets, matrix metalloproteinases (MMPs) and AMP-dependent protein kinase (AMPK), in these pathologies. The final section of this chapter will review current and potential pharmacological approaches to decrease the deleterious action of oxidative stress in the cardiovascular system by modulating the action of the downstream protein targets.

1.1.1 Sources of oxidative stress in cells related to the cardiovascular system

1.1.1.1 Enzymes

1.1.1.1.1 NAD(P)H oxidases

NAD(P)H oxidases were first described in neutrophils, they are membrane bound enzymes that catalyze the one electron reduction of oxygen to form $O_2^{\bullet -}$ using either NADH or NADPH as an electron donor. In the cardiovascular system this enzyme has also been found in smooth muscle cells,[14] fibroblasts,[15] endothelium,[16] and in cardiac myocytes.[17,18] The activation of NAD(P)H oxidases plays an important role in the pathogenesis of cardiovascular diseases such as atherosclerosis, hypertension, heart failure, etc.[2,14,19]

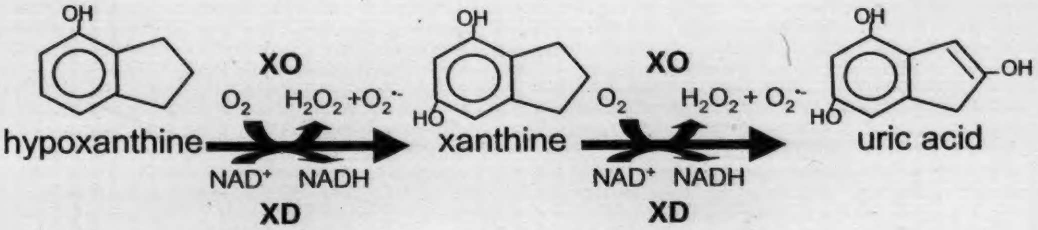
NAD(P)H oxidase requires for its activation the assembly of at least four different subunit proteins. gp91phox (or the homologous NOX1, NOX4, and NOX5) together with p22phox form a protein complex attached to the cell membrane called cytochrome b_{558} which functions as an electron donor. The p21 phox, p67 phox and p47 phox function as regulatory proteins along with the G-protein Rac. The amount of $O_2^{\bullet -}$ produced varies among the different cell types in the cardiovascular system depending on the stimulation by different mechanisms such as tumor necrosis factor-α,[20] platelet derived growth factor,[21] angiotensin II,[22,23] thrombin,[24,25] etc. The whole complex requires attachment to the cell membrane in order to become fully activated. The production of $O_2^{\bullet -}$ does not only produce cytotoxic effects but also plays a role in intracellular signalling pathways.[14]

1.1.1.1.2 Flavoproteins

Flavoproteins are proteins constituted by an isoalloxazine ring that can be reduced via two electron transfer to produce a dihydroisoalloxazine ring. These enzymes employ flavin nucleotides FAD or FADH as cofactors involved in the electron transfer process. This group of proteins include xanthine oxidase, monoamine oxidase and aldehyde oxidase.[26] In the heart only the expression of xanthine oxidase and monoamine oxidase have been reported.

Xanthine oxido-reductase

Xanthine oxido-reductase (XOR) is a cytosolic homodimer protein, each unit 150 kDa.[27] It consists of two interconvertible forms, xanthine dehydrogenase and xanthine oxidase. XOR catalyzes the conversion of xanthine to uric acid in the purine degradation pathway. This metabolic process includes the transfer of six electrons which can be done by one or two electron transfer steps leading to the generation of both $O_2^{\bullet-}$ and H_2O_2 (See reaction 1).[28]



Reaction 1. Generation of $O_2^{\bullet-}$ and H_2O_2 by the xanthine oxidase and dehydrogenase forms of xanthine oxidoreductase.

Activation of XOR plays an important role in the pathogenesis of heart disease. Previously, it has been shown that XOR is increased in isolated rat hearts subjected to cytokine-induced cardiac dysfunction.[9] In addition, activation of XOR is observed in endothelial dysfunction in diseased coronary arteries, hypertension and in animal models of atherosclerosis.[29-31]

Monoamine oxidase

Monoamine oxidase (MAO) is an oxido-reductase widely distributed in various tissues such as neurons, kidneys, gastrointestinal tissue, liver and platelets.[32] However, high expression of MAO-A subtype protein has been reported in the heart.[33] This enzyme catalyses the deamination of different neurotransmitters including noradrenaline, serotonin, adrenaline and dopamine, producing H_2O_2 as a by-product (Reaction 2).[32]

$$RCH_2NH_2 + 2O_2 + H_2O \xrightarrow[MAO]{} RCHO + O_2 + NH_3 + H_2O_2$$

Reaction 2. Generation of H_2O_2 by the MAO catalyzed deamination

1.1.1.1.3 Nitric oxide synthases

Generation of $NO^\bullet$ is very important in the regulation of homeostasis in the cardiovascular system. $NO^\bullet$ plays an important role in the reduction of vascular smooth muscle tone, as well as distinct properties on cardiac muscle including chrono and inotropism as well as the lusitropic properties of the heart.[34] $NO^\bullet$ is synthesized from the conversion of L-arginine and O_2 to $NO^\bullet$ and L-citrulline via nitric oxide synthases (NOS). It is well known that for the production of $NO^\bullet$ via NOS activation, the presence of its substrates L-arginine and oxygen as well as different cofactors, tetrahydrobiopterin, FADH, calmodulin and iron protoporphyrin IX (haem) are necessary.[35] In addition, NOS requires for its activation the binding of two molecules of calcium/calmodulin which increases the flow of electron from NADPH to the reductase flavin domains of NOS.[36,37]

Currently, four isoforms of NOS have been recognized based on the mechanism of activation or the cell type or sub-cellular structure in which they were first recognized: neuronal NOS (nNOS), endothelial NOS (eNOS), inducible NOS (iNOS), and mitochondrial NOS (mtNOS).[38-41] It is well known that all NOS isoforms except iNOS requires calcium for their activation, whereas iNOS is independent of intracellular calcium levels.[42,43] On the other hand, NOS in absence of one of its co-factors (tetrahydrobiopterin) can also synthesize $O_2^{\bullet -}$.[44,45]

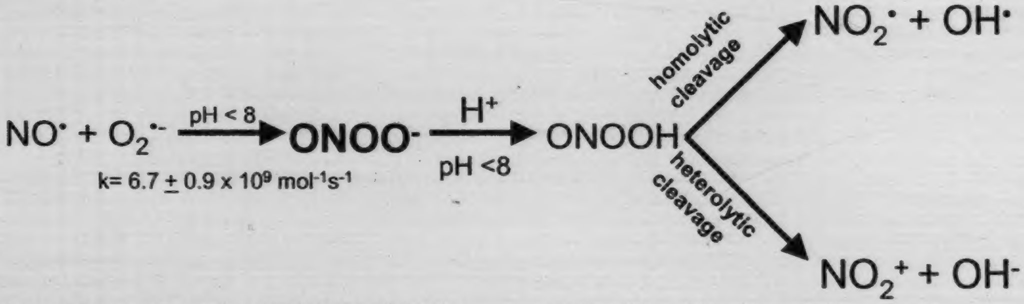
The activation of NOS depends on different signal transduction pathways as well as on post-translational modifications that either increase or decrease the activity of the enzyme. For example, eNOS is regulated by phosphorylation of Ser1177 residue leading to its activation and is inhibited by its interaction with caveolin.[46-48] In addition, recent studies have shown that phosphorylation of eNOS at the Ser-1177 residue by AMP dependent protein kinase results in activation of eNOS in cardiac and endothelial cells.[49,50]

$NO^{\bullet}$ plays different physiological roles in the cardiovascular system, it is a key determinant of vascular relaxation[51,52] as well as being involved in the inhibition of platelet aggregation by mechanisms dependent or independent of guanylate cyclase activation.[53-55] In terms of cardiac performance $NO^{\bullet}$ has shown to reduce heart rate due to different mechanisms including the increased production of cyclic GMP in pacemaker cells[56,57] as well as by increase vagal firing through induction of nNOS localized at the end terminals of vagal nerves.[58] Regarding cardiac contractility $NO^{\bullet}$ has a bimodal effect, based on the amount of $NO^{\bullet}$ synthesized. At relative low concentrations (< 1 μM $NO^{\bullet}$)[59] it can increase cardiac contractility, however, high $NO^{\bullet}$ concentrations clearly decrease cardiac contractility as observed in patients with sepsis (a clinical condition characterized by enhanced $NO^•$ generation through enhanced expression of iNOS and nNOS).[34]

Generation of $ONOO^-$: the main oxidant responsible for the toxicity of $NO^•$

As mentioned before, during stress conditions (such as inflammation, sepsis, etc) there is an increased production of $NO^•$ and $O_2^{•-}$. The reaction of $NO^•$ with $O_2^{•-}$ leads to the formation of highly reactive $ONOO^-$ at a rate ~3.5 times faster than the dismutation of $O_2^{•-}$ by superoxide dismutase (SOD) which acts as a scavenger of $O_2^{•-}$.[35,60]

$ONOO^-$ is a highly oxidant species which can cause modification of proteins, lipids and other biomolecules.[4,60] At pH 7.4 $ONOO^-$ is rapidly protonated to produce peroxynitrous acid (ONOOH) which, then rapidly reacts with other molecules before homolyzing to $NO_2^•$ and $OH^•$ or by undergoing heterolytic cleavage to NO_2^+ and OH^- (Reaction 3).[61]



Reaction 3. Generation $ONOO^-$ and its cleavage at pH < 8 by either homolytic or heterolytic cleavage to highly reactive end products.

It is argued from *in vitro* assays that only 1% of cleaved $ONOO^-$ is converted to $OH^•$ due to the direct reaction of $ONOO^-$ with its molecular targets.[4,60] In addition, in biological tissues the presence of CO_2 concentrations around 1.3-1.5 mM or the CO_2/H_2CO_3 ratio (~25 mM) favours the direct reaction of $ONOO^-$ with CO_2 to produce

$ONOOCO_2^-$, which is rapidly degraded following its protonation to the highly reactive oxidants like $NO_2^{\bullet}$ and $CO_3^{\bullet -}$ (Reaction 4).[4,35,62]

$$NO^{\bullet} + O_2^{\bullet -} \xrightarrow[k = 6.7 \pm 0.9 \times 10^9\ mol^{-1}s^{-1}]{pH < 8} \mathbf{ONOO^-} + CO_2 \xrightarrow[k = 3.0 \times 10^4\ mol^{-1}s^{-1}]{} ONO_2CO_2^- \longrightarrow NO_2^{\bullet} + CO_3^{\bullet -}$$

Reaction 4. Generation and cleavage of $ONOO^-$ in the presence of CO_2 at $pH < 8$.

1.1.1.2. Mitochondrial respiratory chain

Generation of ROS by the reactions of the mitochondrial respiratory chain was first demonstrated by Boveris and Chance in 1973 where they showed the production of $O_2^{\bullet -}$ and H_2O_2 during mitochondrial respiration.[63] The mitochondrial respiratory chain is formed by five protein complexes (I-V). The production of both $O_2^{\bullet -}$ and H_2O_2 has been reported at the level of complex I (NADH dehydrogenase) and II (succinate dehydrogenase).[64] In addition, complex III (Ubiquinol-cytochrome c reductase) also generates O_2^-.[64,65] On the other hand, it has been observed that increased amounts of ROS and $ONOO^-$ at the mitochondrial level inhibit the activity of the different complexes involved in the respiratory chain, mainly complex I, by post-translational modification in its tyrosine residues.[66] In addition, it has been recently showed that increasing concentrations of $NO^{\bullet}$ changes the redox status of complex IV (cytochrome oxidase) facilitating generation of $O_2^{\bullet -}$, mainly during hypoxic conditions (3% O_2).[67]

1.1.1.3 Others

1.1.1.3.1 Peroxisomes

Peroxisomes are organelles identified in cells of the mammalian gut, heart and kidney as well as in lower organisms (yeast) and plants.[68,69] They contain a number of enzymes involved in different metabolic pathways including the oxidation of amino acids or α-hydroxy acids, generating H_2O_2 as an end product. Another important source of H_2O_2 production in the peroxisomes is the β-oxidation of fatty acids.[70] In the heart, it has been showed that they proliferate under stress conditions such as physical exercise, administration of cardiotoxic drugs (adriamicin), the development of hypertension, and after the administration of the cardiotonic drug milrinone.[71] In order to decrease the concentrations H_2O_2 generated in these subcellular structures, peroxisomes have high amounts of catalase (an enzyme which scavenges H_2O_2).

1.1.2 Antioxidant systems

In order to ameliorate the increased production of reactive oxygen or nitrogen species during different metabolic processes, cells contain a highly efficient antioxidant system, which is in charge of the detoxification of these reactive molecules. The antioxidant system is composed by enzymes, proteins, peptides, lipids and other molecules that show some specificity in terms of the reactive molecule to be degraded. In the next paragraphs the role and the mechanisms of antioxidant defense will be described in more detail.

1.1.2.1 Antioxidant enzymes

1.1.2.1.1 Superoxide dismutase (SOD)

Different subtypes of SOD have been located in different cellular compartments; they contain a copper, zinc, manganese, or iron atom in the core. Copper-Zinc (Cu/Zn) SOD is found in the cytoplasm whereas manganese (Mn) SOD is localized in the mitochondria. The ferric form of SOD was reported to be bound to the extracellular side of the plasma membrane. SOD catalyzes the conversion and thus detoxification of $O_2^{\bullet-}$ to H_2O_2 (Reaction 5).

$$2O_2^{\bullet-} + 2\,H^+ \xrightarrow[k=2.7 \times 10^9\ mol^{-1}s^{-1}]{SOD} H_2O_2 + O_2$$

Reaction 5. Dismutation of $O_2^{\bullet-}$ by superoxide dismutase (SOD)

SOD plays an important role in cell homeostasis and their tolerance to oxidative stress. It has been reported that Cu/ZnSOD knockout mice may have a normal lifespan with increased susceptibility to oxidative stress.[72,73] MnSOD knockout mice are not viable due to the development of cardiomyopathy with sudden death.[74,75] The dismutation of O_2^- by SOD occurs at a rate of 2.7×10^9 $mol^{-1}s^{-1}$. On the other hand, it has been shown that $ONOO^-$ inactivates MnSOD, therefore, increasing the oxidative damage by inactivation of this defensive mechanism.[76]

1.1.2.1.2 Catalase

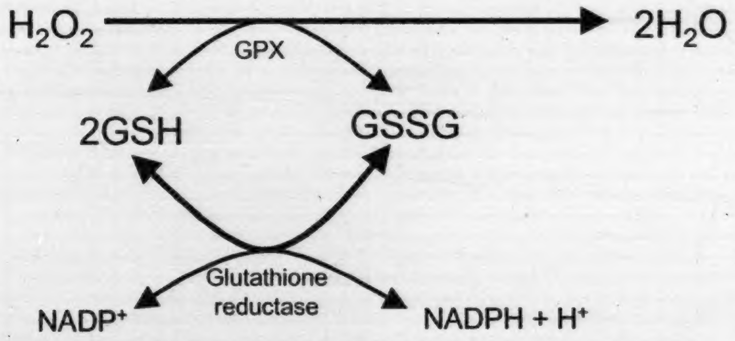
Catalase is present in most eukaryotic cells and it is preferentially localized to the microsomes and the mitochondrial membrane where high concentrations of H_2O_2 are produced. It scavenges H_2O_2 through two different mechanisms such by degrading H_2O_2 to O_2 and H_2O or by acting as a peroxidase (Reaction 6).

$$2H_2O_2 + O_2 \xrightarrow{Catalase} 2H_2O + 2O_2$$

Reaction 6. Detoxification of H_2O_2 by catalase

1.1.2.1.3 Glutathione peroxidase

This enzyme catalyses the degradation of organic peroxides including hydrogen peroxide or lipid peroxides. It is found intracellularly both in the mitochondria and in the cytoplasm. It degrades low concentrations of H_2O_2 (10^{-6} M) at physiological glutathione concentration (10^{-4} to 10^{-3} M), whereas catalase is activated at higher concentrations of H_2O_2 (10^{-3} M) (Reaction 7).[77] Glutathione peroxidase decomposes H_2O_2 at a rate constant 1.5×10^8 $M^{-1}s^{-1}$ and organic peroxides at 3×10^7 $M^{-1}s^{-1}$.[78] In addition, in heart mitochondria, glutathione peroxidase plays a more important role than catalase during oxidative conditions as it contributes approximately 86% of the total scavenging of H_2O_2.[79] Moreover, overexpression of this enzyme protects the mouse heart from ischemia-reperfusion injury as well as prevents left ventricular remodeling and heart failure.[80,81]



Reaction 7. Detoxification of H_2O_2 by glutathione peroxidase (GPX) and restoration of GSH by glutathione reductase

1.1.2.2 Thiols and glutathione

N-acetyl cysteine and glutathione are small molecules that play an important role in protection against oxidative stress. Both thiols contain a highly reactive sulfhydryl (SH) group which is an important target for oxidant species. N-acetylcysteine, a precursor of the synthesis of glutathione, was shown to have beneficial effects against oxidative stress caused by myocardial ischemia and reperfusion in both animals and in humans.[82,83] On the other hand, glutathione supplementation reduced cardiac mechanical dysfunction due to ischemia-reperfusion injury in isolated rat hearts as well as prevented the inhibition of aconitase by $ONOO^-$.[12,84] Moreover, depletion of myocardial glutathione content increases the susceptibility of the heart to oxidative stress.[85,86] On the other hand, glutathione reacts with $ONOO^-$ producing S-nitrosoglutathione, a $NO^\bullet$ donor.[60] Administration of this product was shown to decrease cardiac mechanical dysfunction during ischemia-reperfusion injury.[87,88]

1.1.2.3 α-ketoacids

α-ketoacids such as pyruvate, fumarate, oxaloacetate, malate and lactate, participate in different metabolic processes in the cell.[89] Pyruvate plays an important role in glycolysis as it is converted to lactate in order to produce ATP. In addition, by action of pyruvate dehydrogenase it can be transformed to acetyl CoA in order to be a substrate for the tricarboxylic acid cycle as well as it can be a substrate for the synthesis of amino acids such as valine, leucine and alanine. However, it has been shown that pyruvate has anti-oxidant properties through different mechanisms. First, pyruvate directly reacts with H_2O_2 therefore scavenging this radical, with the resulting end-products acetate, carbon dioxide and water.[90] On the other hand, pyruvate restores the levels of glutathione through increasing the levels of NADPH via the hexose monophosphate shunt which is required for the synthesis of glutathione.[90] There is clear evidence in animals as well as humans that pyruvate and ethyl pyruvate at supraphysiological concentrations (5-10 mM) protect the heart against oxidative damage.[13,91,92]

On the other hand, acetoacetate, a ketone body, also has antioxidant properties by direct interaction with $ONOO^-$ in the presence of CO_2[93] as well as by restoring levels of NADPH and favouring the hexose monophosphate shunt leading to increased glutathione levels in the cell. Acetoacetate (5 mM) was shown to improve the recovery of isolated perfused guinea pig hearts during stunning injury through increasing inotropism and reducing oxidative damage.[94]

1.1.2.4 Vitamins

Vitamins A, C and E react directly with reactive oxygen species such as $OH^{\bullet}$ and lipid peroxides producing a vitamin radical plus H_2O or LOOH respectively. Vitamin E is lipid soluble and reacts with lipid peroxides as well as $OH^{\bullet}$ whereas vitamin C is water soluble. The α-tocopherol moiety of vitamin E is claimed to be the most effective scavenger of lipid peroxides ($k= 23.5 \times 10^5\ M^{-1}s^{-1}$), (Reaction 8).[95,96] In addition, vitamin E can be regenerated by either glutathione or vitamin C.[78]

Studies have shown that supplementation with vitamin E in animal models protect from ischemia-reperfusion injury.[97,98] Recently, it was shown that long term supplementation with vitamin E (400 IU) does not prevent adverse cardiovascular events in patients with vascular disease or diabetes [99]. The same finding was also observed in a large clinical trial of smokers who received either vitamin A or vitamin E as supplement.[100]

$$LOO^{\bullet} + \alpha TOH \xrightarrow[k = 23.5 \times 10^{5}\ M^{-1}s^{-1}]{} LOOH + \alpha TO^{\bullet}\ \text{(a)}$$

$$OH^{\bullet} + \alpha TOH \xrightarrow[k = 23.5 \times 10^{5}\ M^{-1}s^{-1}]{} H_2O + \alpha TO^{\bullet}\ \text{(b)}$$

Reaction 8. Detoxification of lipid peroxides (a) and $OH^{\bullet}$ (b) by α-tocopherol (αTOH). The tocopherol radical $\alpha TO^{\bullet}$ can accept an electron from a different source and produce non radical products.

1.2 ISCHEMIA-REPERFUSION INJURY

Ischemia is defined as cell injury resulting from oxygen deprivation.[101] Myocardial ischemia can result from two conditions: supply ischemia defined as the reduction of arterial blood flow as a consequence of either an obstruction (stenosis or thrombus formation) or increased coronary vascular tone (vasospasm). The other situation is denominated as demand ischemia which results from an inability to provide enough oxygen to the cardiac tissue in response to a stimulus such as exercise or stress that increases the myocardium's oxygen demands.[102]

The first description of myocardial ischemia-reperfusion (I/R) injury was made by Heyndrickx *et al*[103] in 1975 after observing persistence of regional myocardial mechanical dysfunction following reperfusion after brief coronary ligation in dogs. Braunwald and Kloner[104] named this event as myocardial stunning injury. The two important components observed in the setting of myocardial stunning injury include the presence of viable, non-infarcted myocardium with full recovery of contractile function after a period of ischemia and full restoration of coronary flow.

The pathogenesis of I/R injury, especially myocardial stunning, in the heart includes several mechanisms that have been intensively studied in the recent years.[105] They include damage caused by generation of ROS, expression of inflammatory markers, increase in activity of matrix metalloproteinases (MMPs), abnormalities in calcium handling, alterations in contractile and structural proteins, and metabolic derrangements. In the following sections the role of each one of these mechanisms will be more explained at a more extent.

1.2.1 Myocardial I/R injury and ROS

The absence of oxygen supply to the myocardium during ischemia enhances the production of ROS such as $O_2^{\bullet-}$, H_2O_2, $NO^{\bullet}$ and $OH^{\bullet}$.[105-107] Mitochondrial uncoupling may be the main source of $O_2^{\bullet-}$ during ischemia. Upon reperfusion, the reintroduction of oxygen after ischemia leads to a rapid overproduction of $NO^{\bullet}$, $O_2^{\bullet-}$ and $ONOO^-$. These reactive oxygen and nitrogen species may cause damage in the cellular function through their action on proteins, lipids, and deoxyribonucleic acid.[60] The generation of $ONOO^-$ during the reperfusion period following ischemia is one of the causative factors in the ensuing contractile dysfunction.[10,11] In addition, direct administration of $ONOO^-$ decreases cardiac efficiency in isolated rat hearts.[8] Taken together, the overproduction of $ONOO^-$ as well as other ROS such as H_2O_2 during I/R plays a relevant role in the pathogenesis of myocardial stunning following ischemia.[11,13,108,109]

Antioxidant substances which scavenge the excessive amount of ROS as well as the overexpression of enzymes which catalyze the degradation of ROS have been shown to protect the heart from I/R injury. Glutathione, a $ONOO^-$ scavenger, improved the recovery of cardiac function upon reperfusion following ischemia by detoxifying this radical.[12] The same protective effect was observed with scavengers of $OH^{\bullet}$ such as dimethylthiourea, mercaptopropionyl glycine or the iron chelator desferrioxamine in dog hearts subjected to I/R in *in vivo*.[110,111] In addition, mice overexpressing manganese SOD (MnSOD) showed major tolerance to myocardial I/R.[112,113] Furthermore the administration of cell permeable, low molecular weight SOD mimetic compounds decreases the burden of I/R in the heart.[114,115]

1.2.2 Inflammatory markers and myocardial I/R injury

During myocardial I/R a number of inflammatory markers are expressed and have a role in different cellular processes as well as in cellular signalling.[116] These mechanisms include the expression of molecules from several sources (such as neutrophils, monocytes, endothelial cells and cardiomyocytes) that increase neutrophil adhesion such as selectins,[117] intercellular adhesion molecule (ICAM)-1,[118-120] integrins,[117,121] interleukins,[122-125] and complement factors.[123,126-128] The activation of these markers initiates different signalling pathways, producing changes in myocyte function and contributes to the development of myocardial stunning injury. Moreover, blocking the activation of the inflammatory cascade through inhibition of either the production of some interleukins or activation of complement have been demonstrated to have cardioprotective effects in *in vivo* animal models of myocardial I/R injury.[128-130]

1.2.3 Calcium handling and myocardial I/R injury

Alterations in intracellular calcium (Ca^{2+}) handling during myocardial stunning injury in I/R were initially observed by Kusuoka *et al* in isolated ferrets hearts subjected to I/R.[131] Since that first description Ca^{2+} alterations have been reported to play an important role in the setting of myocardial I/R injury.[132] These alterations are characterized by the progressive increase in systolic and diastolic intracellular Ca^{2+} concentrations, decrease in Ca^{2+} transient amplitude, and loss of systo-diastolic excursions due to the secondary rise in Ca^{2+} levels.[132] In addition, increased intracellular Ca^{2+} leads to activation of ATP consuming enzymes with further imbalance between energy supply and consumption[133] as well as activation of Ca^{2+} dependent proteases and phospholipases that normally are inactive at resting concentrations of Ca^{2+},[132] and the triggering of apoptotic processes[134,135]. However, excessive reduction in extracellular Ca^{2+} levels produces deleterious effects in the myocytes. The latter phenomenon is known as the "Calcium paradox".[136-138] Different approaches have been used to diminish the alterations in Ca^{2+} handling during myocardial I/R including the use of Ca^{2+} antagonists (verapamil, nifedipine) and ryanodine (which inhibits Ca^{2+} release from the sarcoplasmic reticulum).[139] In addition in experimental models the overexpression of A_1 adenosine receptor protects hearts from myocardial I/R injury through an improvement in Ca^{2+} handling.[140]

1.2.4 Involvement of contractile and structural proteins during myocardial I/R injury

The contractile apparatus in the cardiac myocytes is formed by thick and thin filaments and the z disc anchoring adjacent sarcomeres. During myocardial I/R myofilaments lose their sensitivity for Ca^{2+} which is reported in terms of reduced maximum calcium-activated force and a shift to the right of $[Ca^{2+}]$-tension relationships.[141-143] In addition, the increase in intracellular Ca^{2+} activates proteolytic enzymes which can degrade contractile proteins. Activation of calpain, a calcium-activated protease, during prolonged times of ischemia ($\geq$ 60 min of duration) may contribute to the degradation of troponin I.[144] In addition, degradation of the natural inhibitor of calpain, calpastatin, is observed during myocardial I/R.[145] However, cardiac myocyte overexpression of calpain surprisingly did not result in any troponin I degradation in mouse hearts.[146] Furthermore, degradation of troponin I is observed in isolated rat heart models of I/R injury.[147,148] In a recent study in piglets subjected to cardiopulmonary bypass, Schwartz *et al*[149] demonstrated that the administration of glucocorticoids prevented the degradation of calpastatin and troponin I, this protective effect could be explained by increase transcription of calpastatin. Conversely, degradation of other sarcomeric proteins including troponin T, α-actinin, myosin heavy chain, myosin light chain, desmin, and spectrin have been proposed to be involved in the pathogenesis of myocardial stunning injury.[150-152]

Preventing the degradation of troponin I by either blocking the activation of MMP-2 (See section 1.3.2) or by using anti-inflammatory agents (glucocorticoids) was shown to reduce the burden of myocardial I/R injury.[149,153] These findings may have potential implications in clinical practice.

1.2.5 Metabolic energetic derangements and myocardial I/R injury

Myocardial I/R injury is related to metabolic changes including an imbalance between energy consumption and energy supply along with an uncoupling between the production of ATP in the mitochondria and its usage for mechanical function upon reperfusion.[154] Glucose transport and glycolysis are increased during I/R in order to meet the demand for ATP in the heart during this condition.[155,156] Changes in glucose metabolism are mediated by mobilization of the glucose transporters (GLUT-4, GLUT-1) to the plasma membrane to enhance glucose transport inside the cell,[157] and phosphorylation of the phosphofructokinase-2 to enhance glycolysis.[158] Consequently, glucose oxidation is markedly reduced during ischemia as a consequence of the inhibition of pyruvate dehydrogenase due to the accumulation of NADH and acetyl CoA in the cytosol resulting from impairment of both Krebs' cycle and electron transport chain.[159] As a result of the Randle cycle, high fatty acid oxidation rates and pyruvate dehydrogenase inhibition leads to low glucose oxidation during reperfusion.[160-162]

Excessive oxidation of fats observed in I/R injury may also cause detrimental effects in mechanical function, likely as a result of increased oxygen consumption.[163] Fatty acid oxidation is enhanced during I/R through a number of mechanisms such as by high plasma levels of fatty acids secondary to cathecolamines release and heparin administration (to avoid blood clotting),[164,165] and by a decrease in malonyl CoA levels which inhibits fatty acid uptake into the mitochondria by modulating carnitine palmitoyltransferase 1.[166]

Different metabolic regulatory mechanisms are triggered by I/R. One such mechanism is the activation of proteins that stimulate or inhibit pathways involved in energetic metabolism such as AMP activated protein kinase (AMPK) which is activated during I/R.[167] This protein acts primarily as a fuel gauge and reduces fatty acid oxidation and stimulates glucose oxidation. However, whether activation of this enzyme is protective or detrimental for mechanical function of the heart in I/R injury remains unresolved. A more detailed review of this protein and its modulation will be described in the next section. Pharmacological interventions have been designed to decrease the metabolic energy derangements during experimental I/R with encouraging results. They include the use of drugs which increase the level of ATP available for cardiac myocyte metabolism during I/R such as trimetazidine, ranalozine and dichloroacetate. El Banani *et al*,[168] showed in isolated perfused rat hearts that trimetazidine enhances ATP synthesis, by increasing glucose oxidation and inhibiting fatty acid oxidation as observed by decreased levels of acyl carnitine. This could be due to an inhibitory effect of trimetazidine on 3-ketoacylcoenzyme A thiolase which plays a role in the later steps of fatty acid oxidation.[169] Ranolazine exhibits a similar effect as trimetazidine and protects against myocardial I/R possibly by enhancing pyruvate dehydrogenase (PDH) activity.[170,171] Dichloroacetate was also demonstrated to protect the heart from ischemic insults.[172,173] It enhances pyruvate oxidation through inhibition of PDH kinase, the enzyme which inhibitis PDH, thereby stimulating glucose oxidation[174] along with some effects on mitochondrial H^+ production.[154,175] Recently, it was shown that the peroxisome proliferator-activated receptor gamma agonists such as the thiazolidinediones or glitazones (rosiglitazone, pioglitazone, etc) used in the treatment of diabetes through their action as insulin sensitizers protect hearts from I/R due to their ability to increase carbohydrate oxidation.[176] However, other studies have shown that these drugs not only have effects on cardiac metabolism but also they have anti-inflammatory properties including the reduction of neutrophil and macrophage infiltration by reducing chemotractant molecules such as intracellular adhesion molecule 1 (ICAM-1) and monocyte chemotractant protein 1 (MCP-1) at the transcriptional level.[177]

1.3. MOLECULAR TARGETS OF ROS IN THE HEART

1.3.1 AMP-activated protein kinase (AMPK)

1.3.1.1 History

The first experimental evidence of the existence of AMPK appeared in 1973.[178,179] However, it was not until 1989 that this protein was given the name of AMPK.[180] AMPK was first identified in the liver where it phosphorylates and inactivates 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and acetyl-CoA carboxylase (ACC). Therefore, it plays an important role in maintaining cellular energy stores. AMPK is activated by either nutrient depletion or impaired generation of ATP, leading to the activation of ATP generating pathways and inhibition of ATP consuming pathways. Based on this premise AMPK is now known as a “fuel gauge” in mammalian cells.[181] Currently, there is significant interest in further understanding the biological roles of AMPK in the cardiovascular system. The next sections will review mechanisms of activation/inhibition of AMPK as well as some insights in the roles of AMPK in the pathophysiology of heart disease.

1.3.1.2 AMPK Structure

AMPK is a heterotrimeric enzyme complex conformed by an α, β, and γ subunit **(Figure 1.1)** which are encoded by different genes. It is reported that in the heart exist two different isoforms of the α subunit (α1 and α2), two β subunits (β1 and β2) and two of the three possible γ subunits (γ1 and γ2).[182]

The α subunit contains not only the catalytic domain but also the regulatory residue threonine-172 (Thr-172). This residue when phosphorylated by upstream kinases leads to the activation of AMPK. The β subunit works as an anchor for the α and γ subunits. However, in skeletal muscle it was shown that high glycogen content inhibits AMPK activity through a poorly understood mechanism that probably involves glycogen interaction with the β subunit.[183,184] The γ subunit contains four cystathione β-synthase domains where AMP is attached, regulating AMPK activity and phosphorylation.[185] Mutations in this subunit have been linked to cardiovascular diseases such as Wolff-Parkinson-White syndrome, cardiac hypertrophy and heart failure.[182]

1.3.1.3 Mechanisms of AMPK modulation

AMPK is activated by elevated concentrations of AMP. Binding of AMP to the γ subunit leads to the conformational change of the AMPK complex. This event allosterically activates the α catalytic subunit, enhances phosphorylation of the Thr-172 residue by the upstream kinases and inhibits the action of protein phosphatase 2C to dephosphorylate the Thr-172 residue **(Figure 1.1)**. It has been shown that physiological concentrations of ATP inhibit AMPK activation, however, this event is not observed in recombinant AMPK lacking the AMP binding site located in the γ subunit.[186]

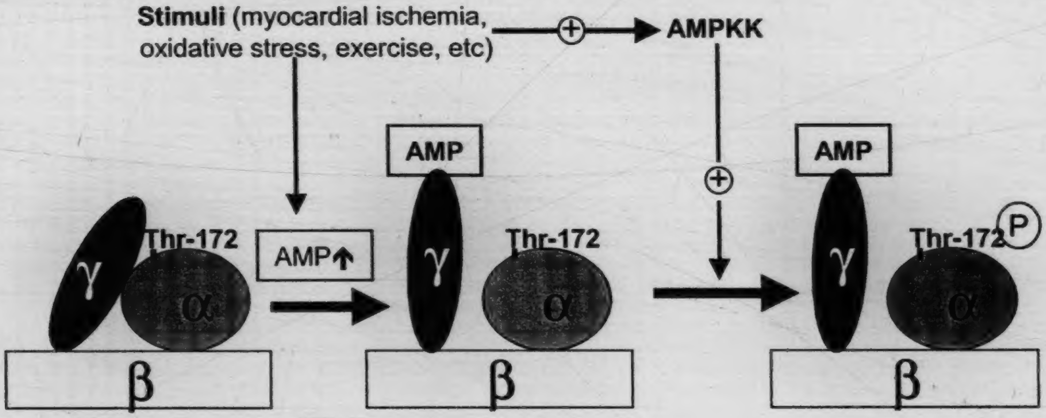

Stimuli (myocardial ischemia, oxidative stress, exercise, etc)
⊕
AMPKK
AMP
AMP
⊕
AMP↑
Thr-172
Thr-172
Thr-172
P
γ
α
β
γ
α
β
γ
α
β

Figure 1.1 AMPK structure and its activation by an increase in AMP levels and/or by direct phosphorylation by action of AMPKK.

As was mentioned before, AMPK is activated by upstream kinases or by AMPK kinase (AMPKK). Altarejos *et al*,[187] showed that AMPKK was activated during periods of either mild or severe ischemia in the isolated rat heart. Moreover, Baron *et al*,[186] also demonstrated the activation of AMPKK in hearts subjected to regional ischemia. In mammalian cells as well as in the liver, LKB1, which is a tumor suppressor kinase, was demonstrated to act as an AMPK kinase.[188,189] However, the identity of the AMPKK(s) that play a role in the activation of AMPK in the heart remains elusive.

Oxidative stress can also cause the activation of AMPK. Choi *et al*,[190] demonstrated that AMPK is activated in NIH 3T3 cells challenged with H_2O_2 (300 μM) due to an increase in the AMP/ATP ratio and phosphorylation of the Thr-172 residue of the α catalytic subunit. Whether the same mechanism occurs in the heart remains unknown.

AMPK can phosphorylate a number of protein targets in the heart including proteins involved in glucose and lipid metabolism, ion channels and nitric oxide synthase (Table 1.1). In addition, chronic activation of AMPK can alter gene expression through interaction with transcription factors, genes related with protein synthesis or by interaction with specific proteins (see Ref. 182).

Table 1.1. Protein targets for phosphorylation by AMPK in the heart

Protein target	Immediate outcome	Biological endpoint	Reference
6-phosphofructo-2 kinase	Enzyme activity↑	Glycolysis↑	158
Acetyl-CoA carboxylase	Enzyme activity ↓	Fatty acid oxidation↑ Fatty acid synthesis↓	191
Malonyl-CoA decarboxylase	Enzyme activity↑	Fatty acid oxidation↓ Fatty acid synthesis↑	192
eNOS	Enzyme activity↑	Production of NO·↑	49
Sodium-voltage gated channels	Channel activity↑	Sodium entry into the cell↑	193

1.3.1.4 AMPK and cardiovascular disease

Recent findings on the biology of AMPK and its protein targets has led to a growing interest in finding the role of this protein in the pathogenesis of cardiovascular diseases. The role of AMPK modulation in different cardiovascular diseases will be discussed in the following paragraphs.

Insulin resistant states show an increase in blood pressure and endothelial dysfunction. Buhl *et al*,[194] showed in insulin-resistant rats that pharmacological activation of AMPK using the adenosine analog 5-aminoimidazole-4-carboxamide-1-beta-Dribofuranoside (AICAR) over the course of seven weeks decreased blood pressure in comparison to obese controls, along with improved lipid and glycemic profiles. In addition, AMPK increases endothelial fatty acid oxidation by inhibiting ACC and stimulates the phosphorylation and activation of eNOS in endothelial cells.[195] Taken together these findings suggest that activation of AMPK can improve endothelial function in diseased states.

AMPK is activated in hearts subjected to I/R.[167] This enhances free fatty acid oxidation in the heart by the phosphorylation of ACC, translocation of the glucose transporter (GLUT-4) to the cytoplasm thereby increasing the flux of glucose inside the cell,[196] as well as increased glycolysis through phosphorylation and subsequent activation of 6-phosphofructo-2 kinase.[158] The consequences of this metabolic modulation in the I/R heart are reflected by the increased production of lactate and protons which have deleterious effects on heart mechanical performance.[197] However, some authors consider that despite the effects of AMPK in fatty acid metabolism during I/R, AMPK activation plays a beneficial role by virtue of increasing glycolysis, glucose uptake and glycogenolysis, thus increasing the available ATP, as well as by indirect mechanisms which include reducing tumor necrosis alpha production and apoptosis.[158,198,199] It is important to clarify that Russell *et al*[198] used low concentrations of fatty acids (0.4 mM oleate) that may have influenced the conclusions of their study. Studies using a pathophysiologically relevant high concentration of fatty acids in I/R tend to show a deleterious role of AMPK activation under these conditions. Therefore, whether AMPK activation during I/R is beneficial or detrimental to the heart remains as an open question and future studies may address this question.

On the other hand, Liao *et al*[200] suggest in a recent study that increased in fasting glucose, insulin resistance and decreased AMPK signaling modulation are implicated in a more rapid development of heart failure in adiponectin-deficient mice.

The role of AMPK in the genesis of hypertrophic cardiomyopathy has been widely described.[201] In addition, mutations of AMPK (either activating or inactivating) have been implicated in the pathogenesis of hypertrophic cardiomyopathy associated to Wolff-Parkinson-White syndrome which is associated with the accumulation of glucogen in the myocytes.[202,203] Interestingly, it has been shown that AMPK can modulate sodium channels by increasing their opening time, which might have consequences in the genesis of arrhythmias.[193]

Based on the various sites of action for AMPK and its protein targets, more knowledge about the mechanisms for its activation and regulation is required. Consequently, this new knowledge may contribute to the design of alternative therapeutical tools for treatment of cardiovascular diseases.

1.3.2 Matrix metalloproteinases

1.3.2.1 Historical perspective

In 1962 Gross and Lapiere demonstrated for the first time the presence of collagenolytic activity in amphibian tissue.[204] After more than 40 years later around 28 different types of MMPs have been identified.[205-207] These enzymes, also called *matrixins*, are zinc-dependent endopeptidases that are best known for their ability to degrade the protein components of the extracellular matrix. MMPs are classified according to their preferred substrate as well as for some structural characteristics or their sub-cellular localization (Table 1.2). MMPs activity has been observed in physiological processes such as bone remodelling, postpartum uterine involution, embryogenesis, and platelet aggregation as well as in pathological conditions such as cancer, wound healing, heart failure, and I/R.[205,208-211] In addition, MMP-1, MMP-2, MMP-3, MMP-9, and many other MMPs are expressed in various cell types relevant to the cardiovascular system (Table 1.3). This section will focus on the mechanisms for both the activation and regulation of MMP-2 (Type IV Collagenase, Gelatinase A or 72kDa Collagenase) and MMP-9 (Gelatinase B, 92kDa Collagenase or Type V Collagenase), given that the former is the MMP found in high concentrations in the cardiovascular system.[212] The latter is an inducible MMP activated during stress conditions such as cytokine stimulation, oncogene products and the presence of ROS.[213-216]

Table 1.2 Classification of MMPs

Group	MMP
Collagenases	
Interstitial collagenase	1
Neutrophil collagenase	8
Collagenase-3	13
Gelatinases	
Gelatinase A, Type IV collagenase	2
Gelatinase B	9
Stromelysins	
Stromelysin-1	3
Matrilysin	7
Stromelysin-2	10
Stromelysin-3	11
Elastases	
Metalloelastase	12
Membrane type-MMPs	
MT1-MMP	14
MT2-MMP	15
MT3-MMP	16
MT4-MMP	17
MT5-MMP	24
MT6-MMP	25

1.3.2.2 Matrix Metalloproteinases 2 and 9

In 1972, Harris and Krane discovered an endopeptidase that was probably MMP-2 or type IV collagenase.[217] In 1978 Sellers *et al*,[218] reported an enzyme with gelatinolytic activity in the culture medium from rabbit bone, which was later given the name of type IV collagenase. MMP-2 has been isolated in mouse tumor cells,[219] rabbit bone,[220] human skin,[221] heart,[212,222] and gingival tissue.[223] MMP-9 (92-kDa-collagenase) was isolated from neutrophils by Sopata and Dancewick.[224] Proteolytic activity against collagen types IV and V was subsequently isolated from rabbit bone culture medium and in a large variety of cell types.[205] After cDNA sequencing it was observed that this enzyme shares homology with the previously identified 72kDa-Collagenase (MMP-2), and that both enzymes degrade native type IV and V collagens and gelatins, but have no activity against collagen type I, laminins, or proteoglycans.[225] In the following section of this chapter, the mechanisms for both activation and regulation of these MMPs will be described.

1.3.2.3 Activation and regulation of MMP-2 and MMP-9

1.3.2.3.1 Protein domains

MMP-2 and MMP-9 have a similar amino acid (aa) sequence and to date the 3D structure of both MMPs has been already constructed.[226] They have similar domains **(Figure 1.2)**, characterized by a *signal peptide domain* (18-30 aa) that acts as a signal sequence guiding the secretion of the enzymes into the endoplasmic reticulum and out of the cells. This domain is not observed in the mature enzyme recovered outside the cell. The *propeptide domain* (80 aa) helps to maintain the enzyme in its inactive form. It has a conserved PRCGVPD sequence positioned directly opposite to the zinc atom of the catalytic centre and coordinates to the catalytic zinc through the cysteinyl–SH group. The modification or removal of the cysteine residue by different processes (oxidation, proteolytic cleavage, mercurial compounds, etc.) activates the enzyme through a phenomenon known as "**the cysteine switch**".[227] MMP-2 and MMP-9 have fibronectin type II domains formed by three repeats of ~ 58 aa localized before the HEXXH zinc-binding site, the fibronectin type II domain function is related to gelatin binding.[228,229] In addition, it has been observed in recombinant MMP-9 that only the second domain is critical for binding to gelatin and domains 1 and 3 were less effective.[230,231] *The catalytic domain* contains a highly conserved sequence **HELGHXXGXXH**, whereby zinc is bound by these three hystidine residues. It is also believed that the presence of the sequence LXXDDXXGI is involved in the stabilization of the active site of MMPs.[232] MMP-9 has a pair of conserved Asp residues at positions 432 and 433 to the C-terminal side of the active centre. The mutation of Asp 432 (D432G), reduces the gelatinase activity by 75% and D433G completely abolishes activity [233]. After this region exists a *hinge or linker region* constituted by a highly variable stretch of aa ranging from 2 to 72 (in MMP-9). *The hemopexin domain* is involved in the binding of TIMPs (tissue inhibitors of metalloproteinases) to MMPs. In the case of MMP-2 this domain also has the ability to bind heparin, which increases the rate of activation eightfold[234] and is required to bind to the cell membrane and to undergo activation at the level of the cell membrane.[235] It has been demonstrated that the binding rate of TIMP-1 to proMMP-9 is much faster when the hemopexin domain is present.[236] MMP-9 has an exclusive type V collagen-like insert localized between the active centre and the hemopexin domain, consisting of 54 aa residues rich in proline whose role is not clear. MMP-2 and MMP-9 are secreted in an inactive form (proMMP-2 and proMMP-9) with an intact propeptide domain, which is further cleaved by different agents to allow for the full activation of the enzyme.

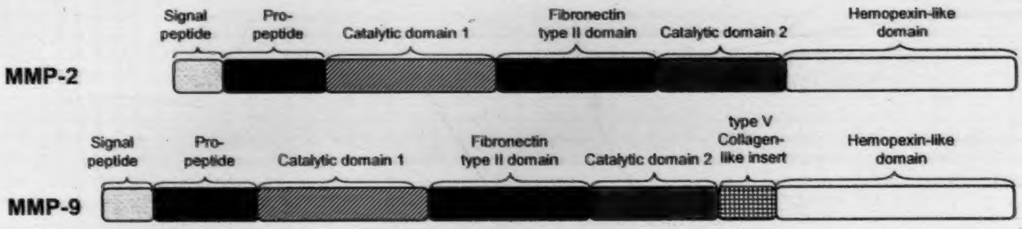

Signal peptide
Pro-peptide
Catalytic domain 1
Fibronectin type II domain
Catalytic domain 2
Hemopexin-like domain
MMP-2
Signal peptide
Pro-peptide
Catalytic domain 1
Fibronectin type II domain
Catalytic domain 2
type V Collagen-like insert
Hemopexin-like domain
MMP-9

Figure 1.2 Schematic representation of protein domains for MMP-2 (upper) and MMP-9 (lower panel).

1.3.2.3.2 Transcriptional mechanisms

The MMP-2 gene is constitutively expressed by many cell types and differs from other MMPs gene family members **(Figure 1.3)**. It is characterized by a lack of TATA or CAAT boxes in the promoter region, absence of the transactivator sequences activator protein 1 (AP-1), polyoma enhancer A binding protein-3 (PEA-3), NFκB sites and upstream TIE (TGF-β inhibitory element).[237,238] Moreover, its transcription is not readily induced by agents such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and is not suppressed but rather slightly upregulated by transforming growth factor β (TGFβ).[239,240] However, recent evidence suggests that in cardiac fibroblast and cardiac myocytes there is a functional AP-1 site which facilitates the induction and transcription of MMP-2.[241] In addition, MMP-2 and MMP- 9 genes contain the activator of protein 2 (AP-2) response element (5'-GCCCAGGC-3'), which contributes to cell-type specific expression.[242] According to its unique transcriptional regulation and the fact that MMP-2 is "constitutively" expressed and not well regulated this gene has been wrongfully characterized as a "housekeeping" gene.[240]

The MMP-9 gene is only constitutively expressed by trophoblasts, osteoclasts, neutrophils, and macrophages. However, it can easily be induced in a variety of cells by cytokines, growth factors and endotoxin. The MMP-9 gene has several control elements

(Figure 1.3) including a TATA-like box present at the 5' flank region but no CCAAT box. There are several GC boxes that can serve as binding sites for Sp1 transcription factors as well as TPA responsible elements (TRE) that can bind AP-1 transcription factors (proteins belonging to the Fos and Jun families). The human MMP-9 gene also possesses an NFκB binding site. This gene also has a consensus sequence for TGFβ whose role in MMP-9 regulation is not completely understood.[243-247] However, a recent study suggests that TGFβ can inhibit the activation of MMP-9 in trophoblasts.[248] Sato and Seiki[243] reported that mutation of AP-1, NFκB and Sp1 in the human promoter of MMP-9 reduces or abolishes the induction by TPA or TNF-α. On the other hand, it is known that dexamethasone and retinoic acid are inhibitors of MMP-9 gene expression.

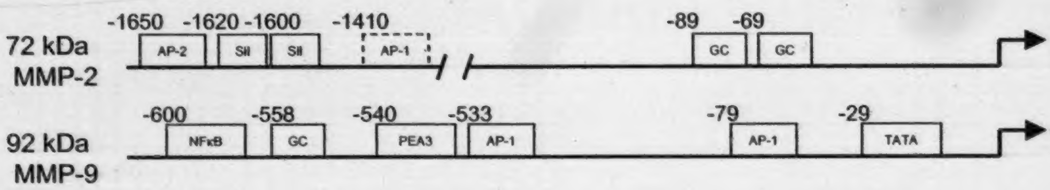


Figure 1.3 Schematic representation of regulatory domains for MMP-2 and MMP-9 genes. The dashed box in the MMP-2 gene represents the AP-1 site for MMP-2 found in cardiac myocytes and fibroblasts

1.3.2.4 Tissue inhibitors of metalloproteinases (TIMPs)

TIMPs are considered endogenous inhibitors of MMPs. Four different types of TIMPs have been cloned and sequenced.[249] The crucial MMPs inhibitory effect of all TIMPs apparently resides at their N-terminal domain involving the tight interaction between the cys1-cys70 disulphide bond. Mutations of this region markedly affects the interaction between TIMP and MMP.[250] TIMPs bind MMPs in a 1:1 stoichiometric ratio.[249] However, each subtype of TIMP has somewhat different affinity for certain

MMPs. It has been observed that TIMP-1 has a high affinity to inhibit MMP-1, MMP-3 and MMP-9, but exhibits lower affinity for MT1-MMP.[236,251,252]

Moreover, TIMP-1 and TIMP-2 are able to bind directly to the hemopexin domain of MMP-9 and MMP-2 therefore maintaining control over the activity of these MMPs. TIMP-1 is easily inducible like its counterpart MMP-9 and TIMP-2 is more constitutive like MMP-2. In addition, TIMP-2 has a dual role in the activation/inhibition of MMP-2: low amounts of TIMP-2 activates MMP-2 by facilitating its proteolytical cleavage by MT-MMP at the cell membrane level[253] but high amounts of TIMP-2 inhibits this enzyme.[254] In terms of affinity, TIMP-2 and TIMP-3 have high affinity to inhibit MT-MMPs but also TIMP-3 is able to bind MMP-2 and MMP-9.[255,256] TIMP-3 is localized primarly in the extracellular matrix and its deficiency has been related to dilated cardiomyopathy and pulmonary diseases (emphysema).[257,258] TIMP-4 is predominantly expressed in the adult heart, but low concentrations have been found in testes, colon, kidney and placenta; similar to TIMP-2 it also has the ability to bind to MMP-2 and MT1-MMP.[259]

On the other hand, other functions of TIMPs aside from their MMP inhibitory actions have been described, such as growth factor activity, steroidogenesis, embryogenesis and which increases the functional complexity of these proteins.[249,260]

1.3.2.5 Mechanisms of activation of the MMPs pro-forms

Physico-chemical activation process. One of the unique properties of the MMPs is that they are activated by proteinases but also by mercurial compounds, thiol reactive agents,[249] iodoacetamide,[224] N-ethylmaleimide,[261] oxidized glutathione,[262] ROS (see below), heat treatment,[263] and brief exposure to acid pH,[264] by means of the cysteine switch mechanism (disruption of Cysteinyl sulphydryl and catalytic Zn^{+2} interaction) **(Figure 1.4)**. However, treatment of proMMP-9 with an organomercurial compound produces an active 68 kDa MMP-9 with a conserved cysteine switch.[265] On the other hand, McLaughlin *et al*[266] reported that the endothelial-cell-stimulating angiogenesis factor dissociated the TIMP-2/MMP-2 complex and the release MMP-2 could not be further inhibited by TIMP-2.

Activation of MMPs by ROS. MMPs can be activated by reactive species such as like HOCl,[267] H_2O_2,[215,216,268] $ONOO^-$,[269-271] and NO_2.[272] MMPs exhibit a particular mechanism of activation by these reactive molecules that differs from the traditional cysteine switch phenomenon. Okamoto *et al*, showed that MMP-1, MMP-8, and MMP-9 can be activated by $ONOO^-$ by the S-glutathiolation of the pro-peptide domain leading to a fully active full length MMP without proteolytic loss of the propeptide domain **(Figure 1.4)**.[271] In addition, it was suggested that activation of MMPs by $ONOO^-$ could be mediated by nitration of tyrosine residues in the hinge region and further unfolding of the pro-MMP[216] or by S-nitrosylation of the cysteine residue in the pro-peptide domain as occurs when MMP-9 is activated by either I/R or nitric oxide donor administration.[273] On the other hand, $ONOO^-$ was shown to inhibit TIMP-1 and TIMP-2 which could increase net gelatinolytic activity in the cells.[274,275] Conversely, the effect of $ONOO^-$ on MMPs activity is biphasic as higher concentrations of $ONOO^-$ have clearly been shown to inactivate MMPs.[269,271]

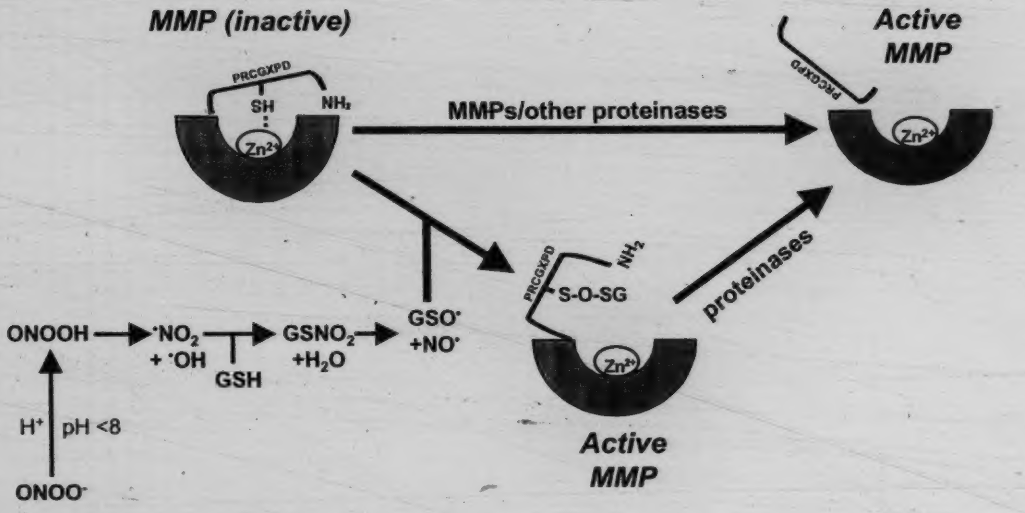

MMP (inactive)
PRCGXPD
SH
NH₂
Zn²⁺
MMPs/other proteinases
Active MMP
PRCGXPD
Zn²⁺
ONOOH
·NO₂ + ·OH
GSH
GSNO₂ +H₂O
GSO· +NO·
PRCGXPD
NH₂
S-O-SG
Zn²⁺
proteinases
Active MMP
H⁺
pH <8
ONOO⁻

Figure 1.4 Mechanisms for activation of MMPs.

Mechanism of cleavage by proteinases and cell surface interaction of proMMP-2. ProMMP-2. It is readily activated by APMA (an organomercurial compound) to a 68 kDa form by cleavage of the Asn80-Tyr81 bond[276,277] but is resistant to endopeptidases including trypsin, thrombin, chymotrypsin, neutrophil elastase, and MMP-3. ProMMP-2 can be activated by MMP-1 and MMP-7.[278,279] Mazzieri *et al*[280] reported that cell bound proMMP-2 can be activated by the urokinase-type plaminogen activator/system, this phenomenon has also been observed for proMMP-9. However, a very significant activation pathway of proMMP-2 is its cell surface activation by MT-MMPs (MT1-MMP, MT2-MMP, or MT3-MMP).[253,281,282] On the cell surface, MT-MMP binds to TIMP-2 which is already bound to proMMP-2 through their respective c-terminal regions creating a ternary complex (MT-MMP/TIMP-2/proMMP-2), where the subsequent activation of proMMP-2 by the closely located free MT1-MMP takes place.

ProMMP-9. This MMP is activated by trypsin, chymotrypsin, kallikrein, and cathepsin G producing an active form between 77-65 kDa. It has also been observed that proMMP-9 is activated by MMP-1, MMP-2, MMP-3, MMP-7, MMP-10 and MMP-13 by a stepwise mechanism. However, there is some discrepancy regarding the activation of proMMP-9 by MMP-1 probably due to differences in the activation conditions.[205]

1.3.3 Matrix metalloproteinases and heart disease

1.3.3.1 Matrix metalloproteinases and myocardial I/R

MMPs are present in the heart and participate in the cardiac matrix remodelling process. They are involved in several physiological processes such as extracellular matrix degradation, cell migration, angiogenesis, remodelling of newly synthesized connective tissue, and the regulation of growth factor activities. However, MMPs also play an important role in the pathogenesis of cardiovascular diseases including myocardial I/R injury, infarct, heart failure, angina, hypertension, and the development of thoracic or abdominal aneurisms. In the following paragraphs the role of MMPs activation mainly in the settings of myocardial I/R or infarct will be discussed in more detail.

The most widely recognized role of MMPs in the heart is related to their action on remodeling of the extracellular matrix.[206] However, recently the degradation of troponin I, a regulatory element of actin-myosin interaction, by MMP-2 was reported during myocardial I/R in the isolated rat heart.[153] The role of MMP-2 on cardiac contractility was recently corroborated by Wang *et al* in papillary muscles from hearts of transgenic mice with a cardiac myocyte specific overexpression of an active MMP-2. They showed impaired contractility of isolated trabeculae measured in terms of increased stiffness and decreased sensitivity to calcium-induced force generation.[283]

As previously explained, MMPs can be activated by ROS as well as by cytokines. Wang *et al*[284] demonstrated in isolated perfused rat hearts the activation of MMP-2 and subsequent irreversible cardiac dysfunction following infusion of $ONOO^-$. In addition, MMP-2 and MMP-9 are activated in cardiac fibroblasts after stimulation with H_2O_2 or generation of $O_2^{\bullet-}$.[215] On the other hand, the administration of pro-inflammatory cytokines to isolated rat hearts impairs mechanical function via the activation of MMP-2 and degradation of troponin I.[285] The activation of MMPs has been demonstrated in different animal models of myocardial I/R.[208,286] Cheung *et al*[208] showed that the activity of MMP-2 released in the coronary effluent peaked 1 min after the onset of the reperfusion period following 20 min ischemia in isolated rat hearts. Furthermore, MMP-2 and an endogenous tissue inhibitor of MMPs (TIMP-4) have been found in the cardiac sarcomere, where MMP-2 was shown to be co-localized with troponin I.[153,287] In a similar study of isolated rat hearts subjected to I/R injury the authors found an imbalance between TIMP-4 and MMP-2 in the sarcomeres of cardiac myocytes leading to an increase of the net gelatinolytic activity inside the cell.[287] Human hearts subjected to I/R injury, as a result of cardiopulmonary bypass surgery grafting for coronary artery bypass show increased activation of MMP-2 and MMP-9 in atrial biopsy tissue within 10 min reperfusion and plasma.[288,289] The level of activation of cardiac MMP-2 and 9 in this setting correlated positively with the maximum degree of post-operatory mechanical dysfunction observed in these patients.[289]

Inhibitors of MMPs were demonstrated to reduce the burden of myocardial I/R injury. Pharmacological inhibition using non-specific MMPs inhibitors (o-phenanthroline or doxycycline, or a neutralizing antibody to MMP-2) improved the recovery of cardiac mechanical function observed during the reperfusion period following ischemia.[153,208,287] In addition, myocardial protection was observed in MMP-9 knock-out mice subjected to *in vivo* myocardial I/R.[290] It has also been reported that classical preconditioning not only reduces contractile dysfunction but also the activation of MMP-2 during I/R.[291] Moreover, inhibition of the expression of low-density lipoprotein receptor (LOX-1) by using an antibody against this protein modulates MMP-1 expression as well as other inflammatory markers, resulting in a protection against myocardial I/R.[292] Administration of transforming growth factor-β decreases contractile dysfunction in rats subjected to coronary ligation for one hour followed by one hour reperfusion by reducing the expression of MMP-1.[293] Recently it was demonstrated in rabbits that pharmacological inhibition of nuclear factor kappa B (NF-κB), which up-regulates MMPs, reduced contractile dysfunction through inhibition of MMP-2 and MMP-9 gene expression in rabbits subjected to cardiopulmonary bypass.[294]

An increase in the activity of MMP-1, MMP-2 and MMP-9 has been reported in human, rat, mouse and porcine hearts after myocardial infarction (MI).[208,295-300] Cheung *et al*[208] reported using a rat heart I/R model that the 72 kDa form of MMP-2 was released into the coronary effluent following ischemia and its concentration peaked within the first minute of reperfusion. This event was directly proportional to the duration of the ischemia period and inversely proportional to the mechanical recovery after 30 min of reperfusion. The administration of MMP inhibitors decreases the early left ventricular dilation 4 days after experimental infarction in mice.[301] In addition, Durcharme *et al*[302] demonstrated in a mouse model of myocardial infarction that in MMP-9 knock-out mice, the post-myocardial enlargement of the left ventricle and collagen accumulation was lower than in wild type mice suggesting that MMP-9 plays an important role in extracellular matrix remodelling after MI. These results were further confirmed by Matsamura *et al*[303] who observed that targeted deletion of MMP-2 in mice or pharmacological inhibition using MMPs inhibitors reduced cardiac rupture in this model. The upregulation of the induction/activation system for MMPs can be also observed in the hearts of patients with heart failure.[211] In addition, high levels of MMP-9 in plasma have been correlated as a prognostic marker for adverse outcome in patients with unstable angina.[304]

Table 1.3 Synthesis of MMPs and TIMPS cells types related to the cardiovascular system (modified from 305).

Cell Type	MMP	Reference
Cardiomyocyte	MMP-2	306
	MMP-3	307
	MMP-9	307
	MT-1 MMP (MMP-14)	308
	TIMP-1	309
	TIMP-2	309
	TIMP-3	309
	TIMP-4	309,310
Cardiac fibroblasts	MMP-1	296,311
	MMP-2	311-315
	MMP-3	312
	MMP-9	313,314
	MMP-13	313
	MT-1 MMP (MMP-14)	312
	TIMP-4	316
Endocardial cells	MMP-2	317
	TIMP-2	318
	TIMP-3	318
Smooth muscle cells	MMP-1	319,320
	MMP-2	321-324
	MMP-3	320
	MMP-9	319,321,324
	MMP-12	325
	MT-1 MMP (MMP-14)	326
	MT3-MMP (MMP-16)	326,327
	TIMP-1	324,328
	TIMP-2	324
	TIMP-3	324
Endothelial cells	MMP-1	329-331
	MMP-2	314,329,331
	MMP-3	329
	MMP-9	314
	MT-1 MMP (MMP-14)	330-332
	TIMP-1	329,333
	TIMP-2	329,333
Platelets	MMP-1	334
	MMP-2	210,335,336
	MMP-9	336
	MT-1 MMP (MMP-14)	335
	TIMP-1	335
	TIMP-2	335
	TIMP-4	337

1.4 EXPERIMENTAL PHARMACOLOGICAL APPROACHES FOR THE PREVENTION OF I/R INJURY

In order to prevent the clinical implications of I/R injury, multiple pharmacological approaches have been tried in experimental models and small clinical trials of patients who undergo coronary artery bypass grafting surgery or other re-vascularization interventions such as percutaneous angioplasty. A number of agents that have been proven effective in clinical trials are now recommended as ancillary therapy during/after reperfusion including type of cardioplegic solution, β-blockers, antithrombotics (heparin and/or low molecular weight heparins), antiplatelets (aspirin, ticlopidine or clopidrogel) and glycoprotein IIb/IIIa inhibitors.[338-340]

Despite the development of these pharmacological strategies to decrease the extent of stunning injury, the potential use of novel pharmacological agents as ancillary therapy for the reduction of stunning injury remains of great interest for both clinicans and scientists. In the following sections the rationale behind potential experimental pharmacological agents aimed at preventing I/R will be described in more detail.

1.4.1 Free radical inhibitors/scavengers

ROS contribute to the pathogenesis of I/R. Therefore, the design of therapeutical tools aimed to either decrease their generation or scavenge them has garnered considerable interest in the pharmaceutical industry. These drugs can be divided into either mimetics of ROS scavenging enzymes or antioxidant substances. In the next paragraphs the properties of these compounds and their protective effects in preventing I/R injury will be discussed in more detail.

During the past several years, much effort has been dedicated to the design of mimetics of ROS scavenging enzymes including superoxide dismutase, catalase and glutathione peroxide.

Superoxide dismutase mimetics are low molecular weight non-peptide cell permeable compounds that were developed following the promising results obtained using SOD from bovine origin in patients with arthritis/inflammation[341,342] and as a co-adjuvant for chemotherapy in cancer patients.[343,344] However, this drug caused immunological reactions because of its bovine origin and its current use is quite restricted. SOD mimetics based on their chemical structure can be classified as manganese (III) metalloporphyrins, manganese (III) salen complexes or manganese (II) pentazamacrocyclic ligand complexes. Metalloporphyrins such as Mn(III) tetrakis benzoic acid (MnTBAP) can scavenge ROS such as $ONOO^-$ and they have been shown to protect against endotoxic shock where not only $O_2^{\bullet-}$, but also $ONOO^-$ is produced.[345] Manganese III salen complexes possess both SOD and catalase-like activity and have been shown to penetrate the mitochondria based on studies showing an increase in lifespan in MnSOD knock out mice.[346,347] The use of these compounds was proven to be protective in a number of experimental models of neurodegenerative diseases,[346,348,349] hemorrhagic or endotoxic shock[350,351] and I/R injury.[352,353] Manganese (II) pentazamacrocyclic ligand complexes are smaller, cell-penetrating molecules with high affinity to SOD and are very selective for $O_2^{\bullet-}$[354] and are effective in preventing the effects of oxidative stress in models of shock[355] and anoxia-reoxygenation.[356,357] However, to the present date stage 2 or 3 clinical trials have not been published using any of these compounds.

Another therapeutical alternative to reduce the increased amount of ROS generated during cardiovascular pathologies is the use of antioxidant molecules that directly scavenge ROS. In this group vitamins, thiols, α-ketoacids and allupurinol are included. The use of vitamins, especially vitamin A, C and E has been widely studied in terms of prevention of adverse outcomes in patients with cardiovascular disease, with contradictory results. Use of vitamin E in animals models of I/R showed either protection[97,98] or no effect against the insult.[358] However, in the light of clinical trials of patients with heart disease a recent large clinical trial did not show any protection of vitamin E in terms of cardiovascular outcomes or development of cancer.[99] On the other hand, vitamin C along with vitamin E supplementation can reduce biochemical markers of oxidative stress in patients who undergo coronary bypass. However, most of these studies showed that the reduction of these markers does not translate into improvement in heart hemodynamics.[359,360] with the exception of the study by Dingchao *et al*,[361] where they demonstrated hemodynamical benefit of high doses of vitamin C after coronary bypass.

The use of thiols such as N-acetyl cysteine or glutathione as scavengers of ROS is promising due to the positive results from studies in animal models of I/R.[12,84,87,88] In the setting of human studies, the use of N-acetylcysteine in a small clinical trial showed benefit in patients with myocardial infarction that underwent reperfusion therapy with streptokinase.[362] However, in patients who had coronary bypass, N-acetylcysteine reduced oxidative stress markers but was not superior to placebo in terms of hemodynamical markers.[363]

Pyruvate and other α-ketoacids protect against oxidative stress in animal studies of I/R via a direct scavenging of ROS and an indirect mechanisms as discussed in a previous section of this chapter.[13,94] Pyruvate supplementation of cardioplegic solution used in patients undergoing coronary bypass showed in a small clinical trial to protect against stunning injury.[91] However, these results need to be explored in a larger series of patients.

1.4.2 MMPs inhibitors

Due to the important role of MMPs in the setting of I/R as well as other cardiovascular diseases, the use of synthetic MMPs inhibitors may be a novel approach for the treatment of patients with these pathologies. There are different types of MMPs inhibitors based on their chemical structure and pharmacological action. They include hydroxamate compounds, tetracyclines and their derivates, and biphosphonates. The properties that a MMPs inhibitor should have to exert its function include: a) a functional group able to chelate the zinc atom in the catalytic domain such as hydroxamic acid, sulfhydril group or carboxylic acid, b) one functional group that facilitates binding to the enzyme backbone through hydrogen bonds and c) at least one chain that undergoes Van der Waals interactions with the enzyme subsite. Hydroxamate compounds have been widely studied as potential anti-cancer drugs. They include the broad spectrum MMPs inhibitors batimastat, marimastat and prinomastat reaching stage of phase III clinical trials. However, due to lack of efficacy in humans (but not in animal models) and secondary effects such as rhabdomyolisis, arthralgia, and joint swelling they are no longer used.[364,365]

Tetracyclines are antibiotics with a long history of use in clinical practice, they include tetracycline, doxycycline, and minocycline. These compounds inhibit MMP activity at concentrations below the IC_{50} required for their antibiotic effect.[366,367] Doxycycline and minocycline have been widely studied in cancer models,[368] periodontal disease[369] as well as in I/R,[208,370-372] showing protective effects against the damage caused by increased MMPs activity. Preliminary studies using subantimicrobial doses of doxycycline (20 mg twice a day orally) showed reduction in inflammatory markers (C-reactive protein and interleukin-6) in patients with a previous acute coronary episode.[373] However, these results should be evaluated in a larger population. Golub *et al*[374-376] produced chemically modified tetracyclines (CMT) synthesized by removing the dimethylamino group from the carbon-4 position leading to an exclusive MMPs inhibitory effect without antibacterical actions. They were shown to be effective in inhibiting MMPs in different models of cancer cell lines but there are no reports on either cardiovascular cells or isolated hearts.[377] Recently, the recommended dose for a CMT (COL-3 or metastat) in patients with solid malignancies in patients with Karposi sarcoma associated to acquired immunodeficiency syndrome was published in ongoing clinical trials.[378,379] These promising results open an avenue for continuing the development of novel compounds to target the activation of MMPs not only in cancer but also in other clinical entities such as cardiovascular disease.

1.4.3 AMPK modulators

From what was mentioned in the section above regarding the important role of AMPK in the pathogenesis of heart disease, modulation of this protein (enhancing or inhbiting) is an alternative means to treat heart disease. 5-aminoimidazol-4-carboxamide-1-β-ribofuranoside (AICAR) is phosphorylated to ZMP (an AMP analogue) which then activates AMPK through the binding of the ZMP moiety to the AMP-binding site of the γ subunit.[182] It is a very useful experimental drug to induce activation of AMPK in isolated cells and organs. However, its use is not recommended in humans due to an analog compound acadesine, which blocks adenosine uptake, had no beneficial effects and even brought more complications in patients subjected to coronary bypass surgery.[380] AMPK can pharmacologically be activated by other compounds such as biguanides (metformin) or peroxisome proliferators-activated receptor γ agonist drugs (thiazolidones) commonly used to treat type 2 diabetic patients.[182,381] The activation of AMPK by the former is independent of the AMP/ATP ratio whereas in the latter seems to be related to a change in this ratio.[381,382] On the other hand, an inhibitor of AMPK, Compound C,[381] was developed. This compound showed to be useful as an AMPK inhibitor in cell culture or *in vivo* models, but its use in isolated organs such as the heart has not been tested.[381,383,384] Further, pharmacological development is required to have new inhibitors of AMPK that can be potentially used in clinical settings.

1.5 OVERALL HYPOTHESIS AND OBJECTIVES

The main objective of this thesis is to explore how oxidative stress induces activation of two specific proteins in the heart, AMPK and MMPs, and to determine implications of these events in the development of cardiac dysfunction. For Chapters 2 and 3 the primary model is the isolated working rat heart subjected to exogenous oxidative stress by infusion of H_2O_2. For Chapter 4 the experimental model is the isolated rat heart perfused in the Langendorff mode subjected to endogenous oxidative stress (I/R) and consequent stunning injury. Chapters 5 and 6, the experimental model employed is the isolated adult rat cardiac myocyte subjected to exogenous oxidative stress by infusion of $ONOO^-$. *My overall hypothesis is that oxidative stress induces activation of two different enzymes (AMPK and MMPs) that contribute through their action on different molecular protein targets to the development of cardiac stunning injury.*

- Cardiac dysfunction caused by H_2O_2 is partially mediated by activation of AMPK and MMP-2. (Chapters 2 and 3)

To the present date, the work aimed to explore the mechanisms by which oxidative stress caused by H_2O_2 mediates the activation of either AMPK or MMP-2 has been done in cells.[190,215,216] It is noteworthy that in the case of AMPK the only study that explored this mechanism was performed in a NIH 3-T3 cell line not relevant to the cardiovascular system. No studies in this regard have been conducted in the isolated rat heart. In the case of MMP-2 the mechanisms for activation of this protein by H_2O_2 have been observed in macrophages-derived from foam cells,[216] vascular smooth muscle cells[216] or cardiac fibroblasts.[215] However, there is no published study which shows direct activation of MMP-2 by H_2O_2 in the isolated rat heart and its implication with contractile dysfunction caused by this reactive species. Therefore, I hypothesize that H_2O_2 induces activation of AMPK as well as MMP-2 and this could contribute to the contractile mechanical dysfunction in the heart.

➢ *AMPK activity in the heart is increased by exogenous administration of H_2O_2.*

- *MMP-2 activity is increased in the coronary effluent after oxidative stress induced by* H_2O_2.
- *The increased activation of either AMPK or MMP-2 is associated with the development of cardiac mechanical dysfunction.*
- *Scavenging* H_2O_2 *by using antioxidants such as pyruvate or preventing the activation of either AMPK or MMPs with pharmacological inhibitors will ameliorate the deleterious effects caused by* H_2O_2 *in the heart.*

- Contractile dysfunction following I/R is mediated by the degradation of myosin light chain 1 by MMP-2. (Chapter 4)

Previous investigations from our lab and others have demonstrated that the activity of MMP-2 is increased and released into the coronary effluent upon myocardial I/R.[153,208,286]In addition, it was demonstrated that cardiac stunning following myocardial I/R is in part due to the degradation of troponin I by MMP-2.[153] Whether MMP-2 degrades other protein targets besides troponin I in the setting of I/R is unknown. On the other hand, degradation of other sarcomeric proteins such as desmin, alpha actinin, and myosin light chain 1 (MLC1) by unknown proteolytical activity in the setting of myocardial ischemia has been reported.[150-152] MLC1 proteolysis could lead to contractile dysfunction due to a decrease in the stability of the myosin neck region which may affect the kinetics of cross-bridge cycling.[385] However, the protease responsible for the degradation of MLC1 is unknown. Thus, I hypothesize that the enhanced activity of MMP-2 during I/R leads to the degradation of other proteins involved in the contractile machinery besides troponin I, such as MLC1.

- *MLC1 is degraded in vitro by MMP-2.*
- *MLC1 has specific regions where it can be degraded by MMP-2.*
- *MLC1 is degraded upon I/R in isolated rat hearts.*
- *Pharmacological inhibition of MMPs prevents the degradation of MLC1 and attenuates cardiac stunning injury.*
- *MMP-2 is associated with MLC1 in the cardiac sarcomere.*

- $ONOO^-$-induced contractile dysfunction is mediated by activation of MMPs in the isolated cardiac myocyte. (Chapters 5 and 6)

In vitro evidence showed that $ONOO^-$ is able to activate MMPs. In addition, in myocardial I/R the production of this ROS is enhanced which can lead to the activation of MMPs which is also observed in this setting.[10,208] Previously it was demonstrated in isolated rat hearts that direct infusion of $ONOO^-$ leads to contractile dysfunction as well as activation of MMP-2.[284] However, this study did not differentiate whether the deleterious effect of MMP-2 is independent from its proteolytic effects on the extracellular matrix. To the date, there are no studies in isolated cardiac myocytes that test whether the inhibition of MMPs protects against cardiac dysfunction caused by a continuous infusion of $ONOO^-$. If this is the case, a study aimed to find a potential molecular target for MMPs in cardiac myocytes after a single bolus of $ONOO^-$ is required Therefore, I hypothesize that contractile dysfunction due to the activation of MMP-2, upon $ONOO^-$ challenge of isolated cardiac myocytes is related to the actions of this protein on intracellular molecular targets.

- *$ONOO^-$ infusion into isolated cardiac myocytes causes contractile dysfunction.*
- *$ONOO^-$ activates MMP-2 in isolated cardiac myocytes.*
- *The detrimental effect of $ONOO^-$ on contractile function is partially attenuated by inhibiting the activation of MMPs.*
- *The protective effects of MMP inhibitors are independent from restoring intracellular calcium homeostasis.*
- *The increased activation of MMP-2 by $ONOO^-$ leads to the degradation of contractile proteins.*

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CHAPTER 2

AMPK ACTIVATION IS PREVENTED BY PYRUVATE IN HYDROGEN PEROXIDE INDUCED CARDIAC INJURY

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2.1 INTRODUCTION

The generation of excessive ROS can lead to oxidative damage and has been implicated in the development of atherosclerosis,[1] hypertension,[2] and heart failure.[3] Cardiac ischemia-reperfusion injury is accompanied by the generation of several ROS such as $ONOO^-$ and H_2O_2.[4-6] The generation of H_2O_2 in cardiac ischemia-reperfusion injury causes cardiac mechanical dysfunction through a number of mechanisms including generation of cardiac arrhythmias, [7,8] changes in intracellular calcium concentration,[9] changes in carbohydrate metabolism,[10] and alterations in substrate oxidation.[11]

AMPK acts as a metabolic sensor or "fuel gauge" in the mammalian cell (reviewed in[12]). This role is fulfilled by the ability of AMPK to phosphorylate key target proteins that control flux through metabolic pathways of glucose uptake, gluconeogenesis, fatty acid synthesis, cholesterol synthesis, fatty acid oxidation, triglyceride synthesis and triglyceride lipolysis.[13-16]

Mutations in the PRKAG2 gene which codifies the AMPK $\gamma 2$ subunit have been described as resulting in ventricular pre-excitation, atrial fibrillation, conduction defects, and cardiac hypertrophy in human patients.[17-19] Furthermore, overexpression of AMPK in cardiac myocytes results in a prolongation of action potential duration through modulation of sodium channel activity and may explain the arrhythmogenic activity in patients with PRKAG2 mutations.[20]

Several cellular stressors including heat shock, hypoxia, exercise, and cardiac ischemia-reperfusion injury result in the activation of AMPK (reviewed in[21]). Consistent with its role as an energy sensor, AMPK is activated by an increase in either the ratio of AMP to ATP[22] or creatine to phosphocreatine,[23] pH,[23] and by phosphorylation on threonine 172 of the alpha subunit by an upstream kinase, AMPK kinase.[24] During myocardial ischemia which stimulates endogenous H_2O_2 biosynthesis,[6] AMPK is rapidly activated.[13] Although both $ONOO^-$ and H_2O_2 have been shown to cause the activation of AMPK,[25,26] whether AMPK activation mediates the adverse effects of H_2O_2 on heart function is unknown.

Several pharmacological approaches to prevent the burden of oxidative stress and subsequent cardiac dysfunction have been investigated. These include novel antioxidants, cell permeable superoxide dismutase mimetics, and pyruvate.[27] Studies have shown that pyruvate has antioxidant properties in experimental models of ischemia-reperfusion and that pyruvate can prevent H_2O_2 mediated cardiac injury.[6,28-30] The antioxidant effects of pyruvate may include direct non-enzymatic reaction with H_2O_2 to produce acetate, CO_2, and H_2O, and restoration of the balance between reduced and oxidized glutathione.[31] However, it is also possible that pyruvate could exert its beneficial effects by potentiating β-adrenergic inotropism, closing mitochondrial permeability transition pores, and enhancing cardiac energetics.[30,31] Whether pyruvate can prevent H_2O_2 mediated cardiac injury by modifying AMPK activity is not known.

In this study we investigated whether activation of AMPK is responsible for mediating H_2O_2-induced reduction in cardiac mechanical function in isolated working rat hearts. We further investigated whether the ability of pyruvate to prevent the H_2O_2-induced reduction in cardiac mechanical function is due to an inhibition of AMPK, and whether pharmacological inhibition of AMPK can prevent the mechanical deficit caused by H_2O_2.

2.2 METHODS

This investigation was done according to The Guide to the Care and Use of Experimental Animals published by The Canadian Council on Animal Care (revised 1993).

2.2.1 Isolated heart preparations

Male Sprague-Dawley rats (250-350g) were anesthetized with an injection of sodium pentobarbital (60 mg/kg, i.p). The hearts were isolated and aerobically perfused as working hearts[32] at their spontaneous heart rate at 9.5 mmHg preload and 70 mmHg afterload, with 110 ml of recirculating Krebs-Henseleit buffer containing 11 mM glucose, 100 μU/L insulin, 1.75 mM Ca^{+2}, 0.5 mM EDTA, and 0.001% bovine serum albumin in the presence or absence of 5 mM of pyruvate (Sigma) in the buffer. After 20 min of equilibration a reading of cardiac mechanical function was taken (this denoted as t=0 min) and immediately thereafter a single bolus of H_2O_2 (300 μM, Sigma) was added to the recirculating perfusion buffer and heart function was monitored for an additional 5 minutes. Cardiac mechanical function was measured by placing ultrasonic flow probes in the left atrial preload and aortic outflow lines for measurement of cardiac output and coronary flow, respectively. Mechanical function was expressed in terms of cardiac work, the product of cardiac output (ml x min^{-1}) X peak systolic pressure (mmHg). Control hearts received only vehicle (ddH_2O). The heart ventricles were then freeze-clamped with

Wollenberger clamps cooled to the temperature of liquid N_2 after the 5 minute perfusion protocol and stored at -80°C for later processing. Ventricular dry wet ratio was measured by taking 50 mg of frozen, powdered ventricular tissue and drying it overnight on a tared piece of aluminum foil in an oven at 100°C overnight and then recorded the dried tissue weight in the morning.

A second group of hearts were challenged with 300 μM H_2O_2 in the presence of either an AMPK inhibitor Compound C (Cmpd C, 10 μM), a kind gift from Dr. Gaochao Zhou (Merck, Rahway NJ, USA), or its vehicle (DMSO, 0.1%, Fisher Scientific) 10 minutes before ending the equilibration period. Cardiac mechanical function was monitored for 5 minutes following the addition of H_2O_2. The hearts were then freeze-clamped in liquid N_2.

2.2.2 Evaluation of high energy phosphates content from heart tissue

Frozen ventricular tissue (100 mg) was powdered in a pestle and mortar cooled to the temperature of liquid N_2 and then was dissolved in 1 mL of ice-cold 6% perchloric acid containing 0.5 mM EGTA. After vortexing the samples they were left on ice for 10 minutes and then centrifuged at 10,000 g at 4°C for 2 minutes. The supernatant was removed and the pH was reset to 5-7 using 1 M K_2CO_3. After sitting on ice for 10 minutes, samples were centrifuged once at 10,000 g at 4°C for 2 minutes. The supernatant was removed for analysis of high energy phosphates by high performance liquid chromatography as described previously.[33] Results are reported as μmol/g dry wet weight heart tissue.

2.2.3 Ventricular homogenate preparation

2.2.3.1 For AMPK activity

50 mg of frozen powdered ventricular tissue was homogenized in ice-cold buffer containing 0.1 M Tris-HCl (pH 7.5 at 4°C), 50 mM NaF, 5 mM Na pyrophosphate, 1 mM EDTA, 1 mM EGTA, 10% w/v glycerol, 1 mM dithiothreitol (DTT), 0.02% sodium azide and a 1:1000 dilution of protease cocktail inhibitor (Sigma P-8340, Oakville, ON). After homogenization for 30 seconds, the mixture was centrifuged at 800 g for 10 minutes. Protein content was measured in the resulting supernatant using the Bradford protein assay[34] using bovine serum albumin as a reference standard.

2.2.3.2 For immunoblotting

Frozen powdered ventricular tissue was homogenized in ice-cold buffer in 50 mM Tris-HCl (pH 7.4) containing 3.1 mM sucrose, 1 mM DTT, 10 μg/mL leupeptin(, 10 μg/mL soybean trypsin inhibitor, 2 μg/mL aprotinin, and 0.1% Triton X-100. Homogenates were centrifuged at 10,000 g at 4°C for 10 min and the supernatant was collected and stored at -80°C until use. Protein content was estimated by Bradford method as described above.

2.2.4 AMPK activity

AMPK activity in the ventricular homogenates was measured by following the incorporation of ^{32}P into the synthetic peptide AMARAASAAALARRR (AMARA, Alberta Peptide Institute).[13] The assay mixture contained 40 mM HEPES pH 7.0, 80 mM NaCl, 0.8 mM EDTA, 1 mM DTT, 0.2 mM [γ-^{32}P]ATP (Amersham), 5.0 mM $MgCl_2$, 0.2 mM AMARA, 8% glycerol, 0.01% Triton X-100, and 2 μg of the ventricular homogenate. Incorporation of ^{32}P into the AMARA peptide was measured at 30^{0}C for 5 minutes. An aliquot of the reaction was then blotted onto phosphocellulose paper (P81, Whatman), washed in 150 mM H_3PO_4 four times and once in acetone. The phosphocellulose was then dried and counted in 4 ml of scintillant. AMPK activity is expressed as pmol ^{32}P incorporated/min mg protein.

An AMPK assay was also carried out as described above, using three representative homogenates from the control group and three from the H_2O_2 group except that 0.1% DMSO or 10 μM Cmpd C in 0.1% DMSO was included in the assay buffer.

2.2.5 Immunoblotting

Ventricular homogenate were diluted in protein sample buffer containing 10% glycerol, 1% β-mercaptoethanol, 2% SDS, 0.1 mg/ml bromophenol blue, and 43 mM Tris (pH 6.8). The samples were boiled for 5 minutes and then subjected to polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions. Samples (15 μg of protein/lane) were resolved by gel electrophoresis using a Mini Trans-Blot Cell (BioRad) in protein reservoir buffer containing 25 mM Tris (pH 8.3), 0.192 M glycine, and 0.1 % sodium dodecylsulfate. Proteins were then transferred to a polyvinylidene difluoride membrane (BioRad) using Towbin's transfer buffer containing 25 mM Tris (pH 8.3), 192 mM glycine, and 20 % methanol. Membranes were blocked at room temperature for 1 hour in 5% bovine serum albumin in Tris-buffered saline (pH 7.6) containing 0.1%

Tween. Membranes were then incubated at 4°C overnight with the phopho-Thr 172-AMPK antibody (Signal Transduction Laboratories) which had been diluted in blocking buffer (tris buffered saline containing 0.01% Tween-20 buffer [TTBS] with 5% milk). The membranes were then incubated in TTBS buffer containing the secondary antibody (goat anti-rabbit) conjugated to horseradish peroxidase at room temperature for 1 hour. Protein expression was visualized using the ECL® Western blot detection kit (Amersham). To control for protein loading, the membranes were stripped and reprobed with β-actin antibody (Santa Cruz Biotechnology), using the same secondary antibody and conditions as used for the immunoblot of phospho-Thr 172-AMPK.

2.2.6 Statistical analysis

Data are presented as means ± SE (n=6 hearts/group) for the functional data, AMPK activity, and nucleotide content. For the measurement of AMPK activity *in vitro* n=3/group. The data were analyzed using GraphPad Prism 3.00 (GraphPad). One-way ANOVA was used to determine the statistical significance between groups. Post-hoc analysis was performed using the Dunnett test. Two-tailed values of $p < 0.05$ were considered significant.

2.3 RESULTS

The administration of 300 μM H_2O_2 to isolated working rat hearts caused a rapid and progressive decrease in cardiac mechanical function, which was observed after a lag phase of 1 minute after the oxidative challenge (Figure 2.1). The maximal decline in mechanical function was observed within 5 minutes after the administration of H_2O_2 and the hearts retained only 20 ± 5% of their baseline cardiac work. The presence of 5 mM pyruvate abolished the reduction in cardiac work mediated by H_2O_2.

The administration of 300 μM H_2O_2 to the hearts resulted in a rapid (within 5 min) and significant increase in AMPK activity compared to control hearts (Figure 2.2). The presence of pyruvate prevented this H_2O_2-induced activation of AMPK. AMPK is activated by an increase in AMP:ATP[22] or creatine:phosphocreatine[23] ratio and by phosphorylation on threonine 172 by its upstream kinase, AMPK kinase.[24] Table 2.1 demonstrates that the H_2O_2-induced activation of AMPK is not accompanied by significant changes in AMP:ATP ratios. However, there was a significant increase in the creatine:phosphocreatine ratio in hearts exposed to H_2O_2 compared to the control hearts and those exposed to H_2O_2 + pyruvate. The H_2O_2-induced activation of AMPK was also accompanied by a significant increase in the phosphorylation of the threonine 172 residue of AMPK, which was prevented by pyruvate (Figure 2.3).

To evaluate whether the activation of AMPK was responsible for the H_2O_2-induced cardiac dysfunction, we perfused another series of hearts in the presence of H_2O_2 and Cmpd C (10 μM, an AMPK inhibitor) or 0.1% DMSO, its vehicle. Cmpd C reduced the loss in cardiac work (Figure 2.4A) in comparison with the group perfused with H_2O_2 alone (Figure 2.1). However, a similar pattern of cardiac work was observed in hearts perfused with vehicle alone. In Cmpd C or DMSO vehicle treated hearts perfused in the presence of H_2O_2 the remaining mechanical function after 5 min was 52 ± 12% and 56 ± 10%, (n=6 per group) respectively. However, there was no statistical difference in cardiac work between the hearts exposed to Cmpd C and those exposed to DMSO vehicle.

Figure 2.4B demonstrates that both Cmpd C and its DMSO vehicle decreased the phosphorylation of the 172-Thr residue of AMPK. Moreover, there was no significant difference in AMPK activity in hearts exposed to Cmpd C or DMSO compared to hearts perfused with H_2O_2 alone (Figure 2.4C). However, since Cmpd C is a reversible inhibitor that acts by binding at the ATP-binding site on AMPK,[35] the allosteric inhibition by Cmpd C would not be expected to be maintained following homogenization. Thus, in order to verify whether Cmpd C was effective at the concentrations used during the heart perfusions, extracts of ventricular homogenates from control perfused hearts, as well as from hearts perfused 5 minutes with 300 μM H_2O_2, were incubated *in vitro* with Cmpd C (10 μM) or its vehicle (DMSO 0.1%). Under these conditions Cmpd C significantly inhibited AMPK activity to 52 ± 2% and 29 ± 3% of the baseline AMPK activity in both control and H_2O_2 treated hearts, respectively. In contrast, 0.1% DMSO had no effect on AMPK activity in the *in vitro* assay (Figure 2.4D).

We also determined whether AMP:ATP and creatine:phosphocreatine ratios were altered in hearts exposed to Cmpd C and/or DMSO. There was no significant difference in the AMP:ATP ratio between the 3 groups. However, hearts exposed to Cmpd C and/or DMSO had significantly reduced creatine:phoshocreatine levels compared to the hearts perfused with H_2O_2 alone ($p<0.05$) (Table 2.1).

2.4 DISCUSSION

In the present study, we demonstrate that acute administration of H_2O_2 to isolated working rat hearts produced a rapid depression in cardiac mechanical function. The reduction in cardiac mechanical function was accompanied by a significant activation of

AMPK, increased AMPK phosphorylation and an increase in the creatine:phosphocreatine ratio. The presence of pyruvate completely prevented the decline in mechanical function, activation of AMPK, increase in AMPK phosphorylation and increase in creatine:phosphocreatine ratio induced by H_2O_2.

The administration of 300 μM H_2O_2 in 60 minute heart perfusion protocols results in a decline of cardiac mechanical dysfunction with a maximal depression at 5 minutes (See Chapter 3). Furthermore, in isolated adipocytes, the peak activation of AMPK occurred 5 minutes after the oxidative challenge with H_2O_2.[26] Therefore, we used isolated working rat hearts exposed to H_2O_2 for 5 minutes to evaluate the role of AMPK activation in H_2O_2-induced cardiac mechanical dysfunction.

Several mechanisms have been described to explain the contractile dysfunction caused by H_2O_2.[7-10,36,37] In this study, we demonstrate that the H_2O_2-induced cardiac dysfunction is accompanied by a significant activation of AMPK. Since an activation of AMPK can result in cardiac arrhythmias,[17-19] altered sodium channel function,[20] and the inhibition of creatine kinase activity,[23] it seems plausible that AMPK could mediate these described effects of H_2O_2 on cardiac contractile function.

To investigate the potential role of AMPK in H_2O_2-induced contractile dysfunction, we used Cmpd C, a reversible inhibitor of AMPK that acts by binding at the ATP-binding site on AMPK. However, while Cmpd C partially restored cardiac function in the presence of H_2O_2, so did its DMSO vehicle control. Cmpd C, but not DMSO, was able to inhibit AMPK activity *in vitro*. DMSO is known to scavenge hydroxyl radical, and concentrations between 10-400 mM reduce the formation of hydroxyl radical in both isolated organs and cardiac myocytes, leading to improvement of physiological parameters and recovery.[38-40] In this study we used a vehicle concentration of DMSO equivalent to 9.1 mM. Therefore, we cannot rule out that the beneficial effects of Cmpd C on cardiac mechanical function may be partially related to a free radical scavenging property of the vehicle. Our results nonetheless suggest that the AMPK cascade is sensitive to oxidative stress and that antioxidant treatment reduces the ability of reactive oxygen species to activate AMPK.

It is not clear from this study how H_2O_2 activates AMPK. The increase in Thr-172 phosphorylation suggests covalent modification by AMPK kinase, the upstream kinase of AMPK. Our laboratory has demonstrated that AMPK kinase is activated during ischemia-reperfusion,[41] however, it is not known whether AMPK kinase is activated by an elevation in the creatine:phosphocreatine ratio as it is by the AMP:ATP ratio. AMPK activation in the H_2O_2 treated hearts, may in part be mediated by an increase in the creatine:phosphocreatine ratio (Table 2.1). That Cmpd C and/or its vehicle DMSO prevented the H_2O_2 mediated rise in creatine:phosphocreatine ratio in the heart supports this possibility.

Pyruvate is not only an important energy substrate but it also has potent antioxidant effects.[31] In animal models, pyruvate improves β-adrenergic inotropism and restores contractile function after direct challenge with H_2O_2.[30,42] Furthermore, in human failing myocardium, high concentrations of pyruvate stimulates a positive inotropic response through improvement of calcium handling and β-adrenergic response and myocardial energetics.[43-45] In this study, we used supraphysiological concentrations of pyruvate that have beneficial effects on cardiac function.[46,47] The ability of pyruvate to prevent H_2O_2-mediated cardiac injury was associated with reductions in AMPK activity.

Questions still remain regarding the role of AMPK activation and alterations in metabolism during H_2O_2-induced oxidative stress. A previous study from our group demonstrated that exposure of isolated working rat hearts for 70 minutes to 450 μM H_2O_2 results in a significant activation in AMPK, and a decrease in cardiac efficiency,[48] this suggests that AMPK activation by H_2O_2 may affect cardiac function by altering the efficiency of the heart to utilize ATP for mechanical work.[48] The possibility that activation of AMPK may only be a marker of depressed mechanical dysfunction cannot be completely ruled out. However, the ability of AMPK to directly modulate ion channel function[20] and the description of AMPK mutations leading to cardiovascular dysfunction[17-19] suggest that AMPK could play a direct role in mediating the mechanical dysfunction induced by H_2O_2. Further studies are required to explore this possibility. Reduction of cardiac exposure to oxidative stress in clinical and interventional situations by using pharmacological strategies such as antioxidants or AMPK inhibitors may reduce the burden of the complications of ischemic injury.

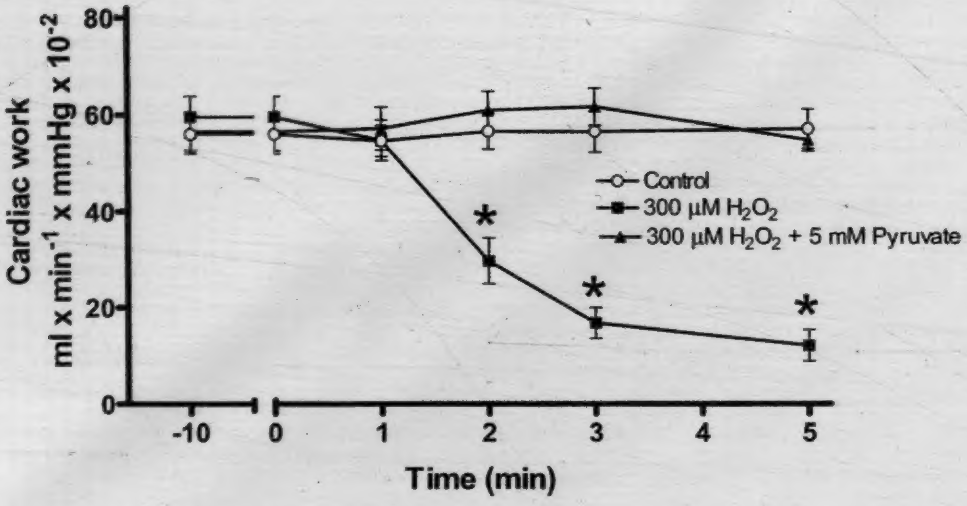

Figure 2.1 Effect of H_2O_2 and pyruvate on cardiac work in isolated working rat hearts. Time course of effects of 300 μM H_2O_2 on cardiac work in isolated rat hearts perfused with Krebs-Henseleit solution in the absence or presence of 5 mM pyruvate. n=6 hearts for each group. * $p<0.05$ vs Control, ANOVA.

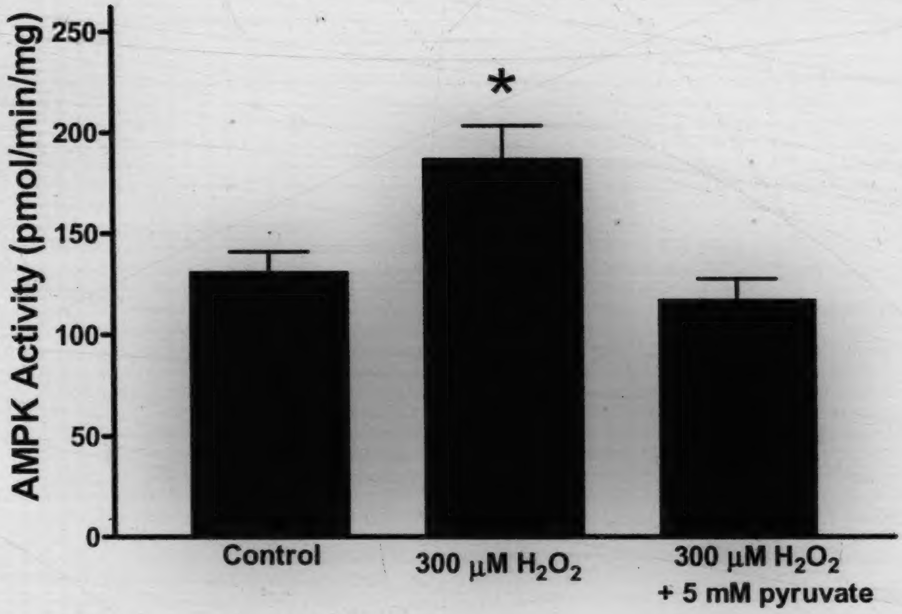

Figure 2.2 AMPK activity in heart homogenates prepared after 5 minute perfusion of hearts with 300 μM H_2O_2. AMPK activity from control perfused hearts (n=6), after 5 min exposure to 300 μM H_2O_2 (n=6), and after 5 min exposure to 300 μM H_2O_2 with 5 mM pyruvate added to the Krebs-Henseleit solution (n=6). * $p<0.05$ vs. control and 300 μM H_2O_2 + 5 mM pyruvate. *(Experiments done with help from LL Atkinson)*

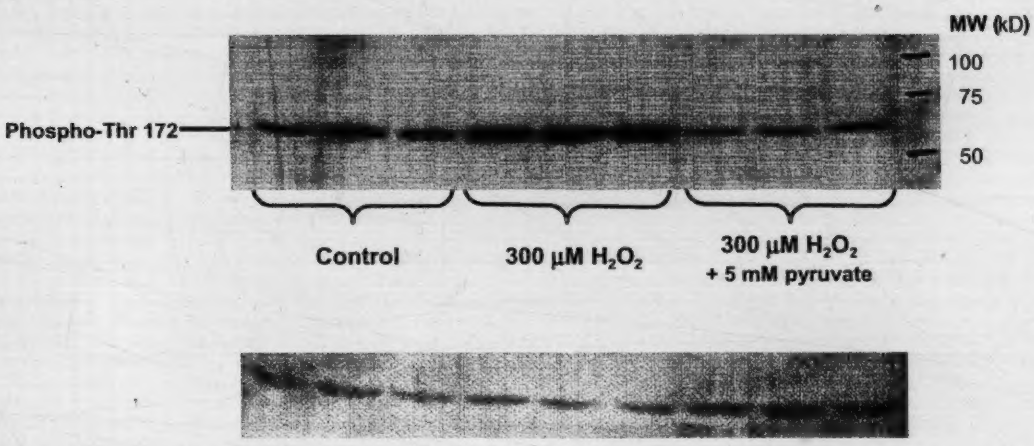
MW (kD)
100
75
50
Phospho-Thr 172
Control
300 μM H_2O_2
300 μM H_2O_2
+ 5 mM pyruvate

Figure 2.3 Thr-172 Phosphorylation of the AMPK α-subunit after 5 minute exposure to 300 μM H_2O_2. Upper panel: representative immunoblot for the phosphorylation of the AMPK α-subunit in control perfused hearts, or those after 5 min exposure to 300 μM H_2O_2 with or without 5 mM pyruvate added to the Krebs-Henseleit solution (n=6). The band of approximately 63 kD indicates the phosphorylated Thr-172 residue of the α-subunit of AMPK. (n=3 hearts shown in each group). The lower panel shows the same membrane probed with β-actin antibody as a loading control. *(Immunoblots done with help from J Sawicka).*

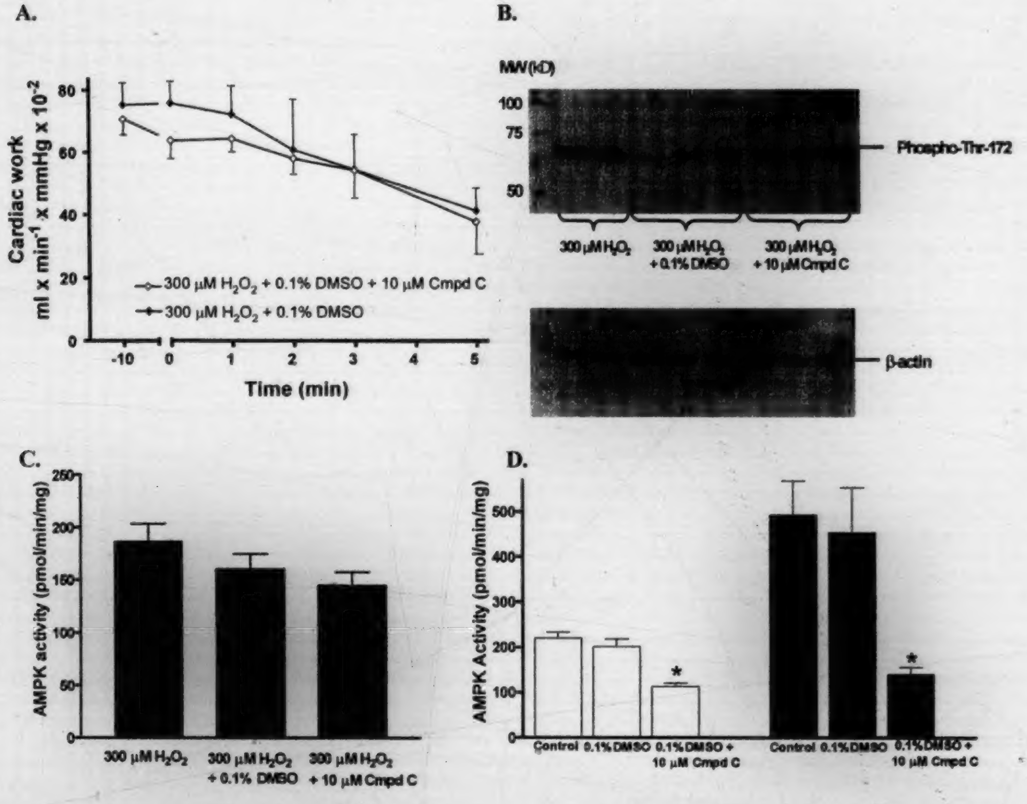

Figure 2.4 Effect of Compound C (Cmpd C,10 μM) or its vehicle (0.1% DMSO) on cardiac work, Thr-172 phosphorylation and AMPK activity. A. Effect on cardiac work in 300 μM H_2O_2 treated hearts. n=6 in each group. **B.** Thr-172 Phosphorylation of the AMPK α-subunit after 5 minute exposure to 300 μM H_2O_2 in the presence of Cmpd C or its vehicle. The lower panel represents the same membrane probed against β-actin as protein loading control. **C.** AMPK activity in hearts perfused 5 min with 300 μM H_2O_2 in the presence of either Cmpd C or its vehicle (n=6 each). **D.** AMPK activity in heart homogenates incubated with either Cmpd C or its vehicle. Hearts were perfused for 5 min alone (Control, open bars) or with 300 μM H_2O_2. (Closed bars, n= 3 for each). They were then homogenized and incubated *in vitro* with Cmpd C or its vehicle for assay of AMPK activity. * $p<0.05$ vs. Control. *(AMPK activity measured by LL Atkinson).*

Table 2.1 High-energy phosphates concentration in heart after 5 minutes exposure to 300 μM H_2O_2[¶]

Group	ATP	ADP	AMP	AMP:ATP ratio	PCr	Cr	Cr:PCr ratio
Control	23.0 ± 3.1	11.8 ± 1.7	3.1 ± 0.9	0.18 ± 0.08	48.6 ± 6.1	66.6 ± 7.8	1.4 ± 0.1
300 μM H_2O_2	24.2 ± 3.0	12.0 ± 2.7	4.3 ± 0.5	0.19 ± 0.02	36.2 ± 7.1	79.7 ± 8.7	2.7 ± 0.7*
300 μM H_2O_2 + 5 mM pyruvate	32.6 ± 5.5	12.3 ± 3.5	3.8 ± 1.1	0.12 ± 0.04	98.4 ± 18.2*	78.5 ± 14.2	0.8 ± 0.1
300 μM H_2O_2 + 0.1% DMSO + 10 μM Cmpd C	19.1 ± 1.4	8.0 ± 0.6	2.4 ± 0.2	0.13 ± 0.02	51.3 ± 6.3	59.3 ± 4.1	1.2 ± 0.2§
300 μM H_2O_2 + 0.1% DMSO	17.2 ± 1.0	7.3 ± 0.4	2.3 ± 0.3	0.14 ± 0.03	43.9 ± 2.0	56.3 ± 1.8	1.3 ± 0.1§

[¶] Concentration of high-energy phosphates is given in μmol/ g dry wet weight

* $p< 0.05$ vs Control, n= 6 hearts per each group; §$p< 0.05$ vs H_2O_2. *(HPLC performed by K Strynadka)*

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CHAPTER 3

HYDROGEN PEROXIDE CAUSES CARDIAC DYSFUNCTION INDEPENDENT FROM ITS EFFECTS ON MATRIX METALLOPROTEINASE-2 ACTIVATION

This work is submitted for publication: León H, Sawicka J, Schulz R. Hydrogen peroxide causes cardiac dysfunction independent from its effects on matrix-metalloproteinase-2 activation. *Canadian Journal Physiol. Pharmacol.*

3.1 INTRODUCTION

Cardiovascular disease is the leading cause of morbidity and mortality in the world.[1] It is well known that many types of cardiovascular diseases are characterized by an increase in oxidative stress.[2] In addition, in I/R injury in the heart it was observed that ROS are produced upon reperfusion, exacerbating cardiac dysfunction.[3-5] Increased production of these oxidant species contributes to the initiation of proinflammatory events, including the activation of MMPs, suggesting that they may be effectors of this type of injury.[6-9] MMPs are proteases best known for their actions in extracellular matrix remodelling. Recently, we have reported that $ONOO^-$ administration causes cardiac dysfunction through the activation and release of MMP-2.[9] Furthermore, MMP-2 is rapidly activated during I/R and it is able to degrade troponin I and MLC1, proteins which are involved in the regulation of the contractile machinery.[10,11] Therefore, one mechanism underlying the loss of cardiac function in I/R may be the enhanced activation of MMP-2 by ROS.

H_2O_2 is a ROS that causes cardiac dysfunction[12-14] by several mechanisms including generation of cardiac arrhythmias,[15,16] changes in intracellular calcium concentration,[17] alteration of carbohydrate metabolism,[18,19] and activation of proteins involved in cardiac metabolism such as AMPK.[20] However, recently Siwik *et al*[8] reported that in cardiac fibroblasts H_2O_2 activates MMP-2, MMP-9, and MMP-13, and that the increase in these MMPs activities may have pathophysiological implications in this kind of injury. However, whether the activation of MMPs by H_2O_2 relates to the mechanical dysfunction resulting from I/R remains unclear.

In order to prevent the burden of oxidative stress not only in I/R injury but also other clinical settings, different pharmacological approaches have been studied including novel antioxidants such as cell permeable superoxide dismutase mimetics (to increase the degradation of $O_2^{\bullet -}$).[21] Pyruvate, a well-known antioxidant, prevented the production of ROS, and the consequent reduction in cardiac performance in models of cardiac I/R injury, as well as after direct administration of H_2O_2.[5,12,22] Pyruvate was shown to be able to scavenge H_2O_2 in both *in vitro* as well as in isolated rat hearts. This was observed by both a reduction in a ROS-induced chemiluminiscent signal *in vitro* and by a decrease in electron spin resonance signal after adding pyruvate to the buffer of hearts challenged with H_2O_2.[5,12,22] The protective effect of pyruvate may be due to its antioxidant properties causing the restoration of the glutathione/glutathione disulfide ratio, potentiation of β-adrenergic inotropism, closure of mitochondrial permeability transition pores, or enhancement of cardiac energetics.[23,24] Whether pyruvate prevents H_2O_2-mediated cardiac injury by preventing the release and activation of MMPs in the heart, as an alternative mechanism, remains unknown.

We therefore hypothesize that in isolated rat hearts, H_2O_2-mediated cardiac dysfunction is caused by an increase in MMP-2 activity which may be prevented by either scavenging H_2O_2 or by pharmacological inhibition of MMPs.

3.2 METHODS

This investigation was done according to the recommendations given by the Guide to the Care and Use of Experimental Animals published by The Canadian Council on Animal Care (revised 1993).

3.2.1 Isolated heart preparations

Male Sprague-Dawley rats (250-350 g) were anesthetized with an injection of sodium pentobarbital (60 mg/kg i.p.). The hearts were isolated and aerobically perfused at their spontaneous heart rate as working hearts with 110 mL of recirculating Krebs-Henseleit buffer containing 11 mM glucose, 100 µU/L insulin, 1.75 mM Ca^{+2}, 0.5 mM EDTA, and 0.001% bovine serum albumin in the presence or absence of 5 mM of pyruvate in the buffer. After 20 min of equilibration in the working mode, a single bolus of stabilized H_2O_2 solution (containing 0.5 ppm stannate and 1 ppm phosphorus, Sigma, Oakville, ON) was added to the perfusion buffer to reach a final concentration of 300 µM H_2O_2. The changes in cardiac function measured as cardiac work (Chapter 2) were followed for 60 minutes. Perfusate samples (2 mL) were collected two minutes before H_2O_2, and at 1, 3, 5, 10, 30 and 60 minutes after the intervention. The hearts were freeze-clamped in liquid N_2 after the perfusion period and stored at –80°C for later analysis. Perfusate samples were kept on ice until further processing.

A second series of hearts was perfused under the same conditions with 300 µM H_2O_2, with or without 5 mM pyruvate. The hearts were freeze-clamped in liquid N_2 5 min after adding H_2O_2.

Two separate series of hearts were perfused in order to test the effects of MMPs inhibitors on cardiac function following challenge with 300 µM H_2O_2. Hearts were challenged with 300 µM H_2O_2 in presence or absence of either doxycycline (75 µM) or Ro 31-9790 (3 µM) administered 10 min before adding H_2O_2. Parallel experiments were performed using their respective vehicles (water for doxycycline and 0.006% DMSO for

Ro 31-9790). The hearts were freeze-clamped in liquid N_2 at the end of the 60 min perfusion protocol.

3.2.2 Preparation of heart homogenates

Frozen ventricular tissue was powdered with a pestle and a mortar cooled to the temperature of liquid N_2, and then manually homogenized in ice-cold 50 mM Tris-HCl (pH 7.4) containing 3.1 mM sucrose, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, and 2 μg/ml aprotinin. The homogenate was centrifuged at 8500 g at 4°C for 5 min, and the supernatant was collected.

3.2.3 Measurement of MMPs activity by gelatin zymography

Gelatin zymography was performed as described.[25] Perfusate samples (1 μg/lane) or heart extracts (20 μg/lane) were applied to 8% polyacrylamide gel copolymerized with 2 mg/ml gelatin. After electrophoresis, gels were rinsed three times for 20 minutes each with 2.5 % Triton X-100 in order to remove SDS. Then gels were washed with incubation buffer containing 50 mM Tris-HCl (pH 7.6), 5 mM $CaCl_2$, 150 mM NaCl and 0.05 % NaN_3 for 20 min each at room temperature and then incubated in incubation buffer at 37°C for 36 or 48 hours for perfusate and heart extract samples, respectively. Conditioned culture medium from the human fibrosarcoma cell line HT-1080 (American Type Culture Collection, Rockville, MD) was used as standard for MMP-2 and MMP-9 activity. The gels were stained in 2% Coomassie Brillant blue G250, 25% methanol, and 10 % acetic acid for 2 hr, and then destained for 1 hr in 2% methanol/4% acetic acid. Zymograms were scanned using a calibrated densitometer GS 800 (BIO-RAD), and the band intensities were analyzed by densitometric analysis using the SigmaGel software (Jandel).

3.2.4 Evaluation of high-energy phosphate content from heart tissue

Frozen ventricular tissue (100 mg) was powdered in a pestle and mortar cooled to the temperature of liquid N_2 and then was dissolved in 1 mL of ice-cold 6% perchloric acid containing 0.5 mM EGTA. After vortexing the samples they were left on ice for 10 minutes and then centrifuged at 10,000 g at 4°C for 2 minutes. The supernatant was removed and the pH was reset to 5-7 using 1 M K_2CO_3. After sitting on ice for 10 minutes, samples were centrifuged once at 10,000 g at 4°C for 2 minutes. The supernatant was removed for analysis of high energy phosphates by high performance liquid chromatography as described previously.[26] Results are reported as µmol/g dry wet weight heart tissue. The dry to wet ratio was calculated as mentioned in section 2.2.2.

3.2.5 Statistical analysis

Data are expressed as means ± SE. One-way ANOVA followed by post-hoc analysis using Tukey's multiple comparison test was used for statistical analysis to compare vs. Control. One-way repeated ANOVA followed by post-hoc analysis using Dunnett's multiple comparison test was used for statistical analysis of hemodynamical parameters within the same group vs. the baseline value. $p< 0.05$ was determined as criterion for statistical difference.

3.3 RESULTS

3.3.1 H_2O_2 impairs cardiac mechanical function

Isolated rat hearts were perfused for 60 min to evaluate the effects of 300 µM H_2O_2 on cardiac function in the presence or absence of pyruvate. 300 µM H_2O_2 caused cardiac dysfunction that was maximal 5 min after the administration of H_2O_2 (Figure 3.1A). The administration of pyruvate markedly reduced cardiac mechanical dysfunction caused by H_2O_2, as cardiac work was similar to control values (Figure 3.1A). In terms of coronary flow, hearts subjected to 300 µM H_2O_2 exhibited a progressive increase in coronary flow which was significantly different from the control group after 10 min of the H_2O_2 challenge. Hearts challenged with 300 µM H_2O_2 in the presence of pyruvate showed an increase in coronary flow which was not statistically different from Control hearts (Figure 3.1B).

3.3.2 H_2O_2 induces release of MMP-2

There was a significant increase in the release of 72 kDa MMP-2 into the perfusate which reached a peak 5 minutes after 300 µM H_2O_2 (Figure 3.2). At 10 minutes the MMP-2 level was still elevated however it was not statistically significant (p=0.07). Pyruvate not only prevented cardiac dysfunction but also reduced the release of 72 kDa MMP-2 into the perfusate (Figure 3.2A and 3.2B).

In hearts perfused for 60 minutes with H_2O_2 there appeared to be a reduction in 72 kDa MMP-2 activity vs. Control, however this was not statistically significant (Figure 3.3). We did not observe changes in the tissue level of 72 kDa MMP-2 activity during the maximal depression of function seen at 5 minutes after the administration of 300 µM H_2O_2 in the presence or absence of pyruvate (data not shown).

3.3.3 The protective effect of pyruvate is independent of changes in high energy phosphate content in hearts exposed to H_2O_2

Oxidative stress can change the level of high-energy phosphates, and this may be prevented by antioxidants such as pyruvate, which also functions as a substrate in cardiac metabolism. Therefore, we evaluated the high-energy phosphate content of heart tissue freeze-clamped after 60 minutes of perfusion. No differences in the levels of AMP, ADP or ATP among the three studied groups were detected (Table 3.1). Pyruvate hearts showed a significantly lower AMP/ATP ratio in comparison with control hearts ($p < 0.05$). No differences in phosphocreatine, creatine or the ratio of the two were observed among the studied groups.

3.3.4 MMPs inhibitors do not protect against H_2O_2-mediated mechanical dysfunction

The administration of 75 μM doxycycline before exposure to 300 μM H_2O_2 did not prevent cardiac mechanical dysfunction in comparison to 300 μM H_2O_2 alone (Figure 3.4A). A higher concentration of 100 μM doxycycline was also not protective (n=4, data not shown). Doxycycline itself had no effects on cardiac mechanical function or coronary flow during the 60 min perfusion protocol (data not shown, n= 4).

We compared the results with doxycycline, with that of a different MMPs inhibitor (Ro 31-9790) in this model of cardiac injury. Cardiac mechanical function in hearts exposed to 3 μM Ro 31-9790 without H_2O_2 remained stable during the perfusion protocol (Figure 3.4B). Ro 31-9790 did not prevent the reduction of cardiac work upon H_2O_2 challenge (Figure 3.4B). The DMSO vehicle control for Ro 31-9790 had similar effects on mechanical function as hearts subjected to the compound in the presence of H_2O_2.

3.3.5 MMPs inhibitors do not change MMP-2 level in the heart

We measured 72 kDa MMP-2 activity in homogenates of hearts treated with either doxycycline or Ro 31-9790. In homogenates from hearts subjected to H_2O_2-induced injury in the presence of 75 μM doxycycline, the activity of 72 kDa MMP-2 was not changed (Figure 3.5A). Surprisingly, MMP-2 activity of hearts subjected to H_2O_2 in the presence of Ro 31-9790 was significantly higher than in hearts treated with H_2O_2 alone (Figure 3.5B). DMSO vehicle did not change 72 kDa MMP-2 activity vs. H_2O_2 alone. It was lower than the result with Ro 31-9790 but this did not reach statistical significance (p=0.07).

3.4 DISCUSSION

In this study we demonstrated that 300 μM H_2O_2 caused a depression of cardiac mechanical function with incomplete recovery. This phenomenon was accompanied by the release of increased 72 kDa MMP-2 activity into the perfusate that peaked at the time of maximal cardiac depression without a significant change in activity of MMP-2 in the tissue. Pyruvate prevented both the cardiac dysfunction and the increased release of MMP-2 activity into the perfusate. However, pharmacological inhibition of MMPs by two different MMP inhibitors with distinct molecular structures did not prevent cardiac mechanical dysfunction caused by H_2O_2. Despite our inability to draw conclusions from the results regarding the roles of MMPs in this model, this study represents significant progress in terms of technical challenges of testing the effects of pharmacological agents in the context of H_2O_2 challenged hearts such as setting their effective working concentrations, buffer composition and exploring the reproducibility of the model.

The role of H_2O_2 in MMPs activation in the cardiovascular system has not been widely studied, especially in cardiac muscle. Siwik *et al*,[8] showed that MMP-2, MMP-9, and MMP-13 are activated in neonatal and adult cardiac fibroblast by H_2O_2 and by a $O_2^{\bullet-}$ generating system. They found that a low concentration of H_2O_2 (5 µM) was able to activate both "pro" and active forms of these MMPs as well as to inhibit collagen synthesis in this cell culture model. However, they did not test the functional consequences or the effects of MMPs inhibition on this system as we did in our study using higher concentrations of H_2O_2 that were required to have a significant effect on cardiac function. Recently, it was shown that 100 µM H_2O_2 induces activation of epidermal growth factor-like activity in cardiac fibroblasts and cardiac myocytes via a mechanism that includes MMPs activation. MMP inhibitors blocked this pathway upstream of the activation of the epidermal growth factor receptor.[27] H_2O_2 can induce transcriptional upregulation of MMP-1 and MMP-2 suggesting that a feedback loop may amplify this effect.[28,29] However, acute effects of H_2O_2 in the activation of MMPs in the isolated organ have not been tested and we did not address the effect of H_2O_2 at the transcriptional level of MMPs in the heart.

H_2O_2 can cause cardiac dysfunction through diverse mechanisms including action on ion channels,[30-32] calcium homeostasis,[33,34] metabolism,[18-20] and through irreversible oxidative modification of contractile proteins.[35,36] In our study we were able to observe that pyruvate prevented cardiac mechanical dysfunction caused by H_2O_2. In this study, no differences in nucleotide content between hearts challenged to H_2O_2 in the absence or presence of pyruvate were observed, suggesting no direct effect of pyruvate in mantaining high-energy phosphate levels. However, cardiac dysfunction was not rescued by MMPs inhibition. The concentrations of the MMP inhibitors used were shown to be protective against cytokine-induced cardiac mechanical dysfunction and I/R injury,[25,37] suggesting that they are effective in this system. However, we have no way of directly testing the degree of MMP blockade in the working heart. The protection that was seen using pyruvate is likely mediated by the H_2O_2 scavenging effect of pyruvate.[5,12,22]

Our study raises the possibility that DMSO concentration (546 μM) might form a toxic compound in combination to H_2O_2 that affected cardiac function (Figure 3.4) or that it was not capable of scavenging $OH^{\bullet}$ radicals which is normally achieved at concentrations > 10 mM.[38-40] To our knowledge there are not reports in the literature that support or reject the first possibility.

Interestingly, this model of exogenously induced oxidative stress is different from the one caused by $ONOO^-$. Previously we showed that isolated working rat hearts subjected to continuous infusion of 40 μM $ONOO^-$ caused an irreversible contractile dysfunction which was significant 30 min after starting the infusion.[41] In this study we observed that H_2O_2 had a rapid deleterious action on cardiac function (peak at 5 min), with partial recovery at the end of the perfusion protocol. However, one limitation of our study is the fact that the magnitude of mechanical dysfunction after H_2O_2 was not the same in all the series of perfused hearts. This could be as a consequence that the different series of experiments were perfused at different times (months to years of interval between series). In addition, Wang *et al*[9] in the Langendorff perfused heart showed that either MMP inhibition or scavenging of $ONOO^-$ with glutathione protected the heart from $ONOO^-$-induced cardiac dysfunction. The release of MMP-2 upon H_2O_2 challenge observed in this study is less pronounced than in hearts exposed to $ONOO^-$, suggesting that H_2O_2 mediated activation of MMP-2 is less important from the pathophysiological standpoint than when the heart is challenged with $ONOO^-$.

In conclusion, the present study shows that H_2O_2 in the isolated perfused heart causes cardiac mechanical dysfunction through MMP-independent mechanisms. The use of antioxidants or inhibition of the generation of ROS such as H_2O_2 may have a therapeutic effect in the setting of acute implications of oxidative stress in cardiac disease.

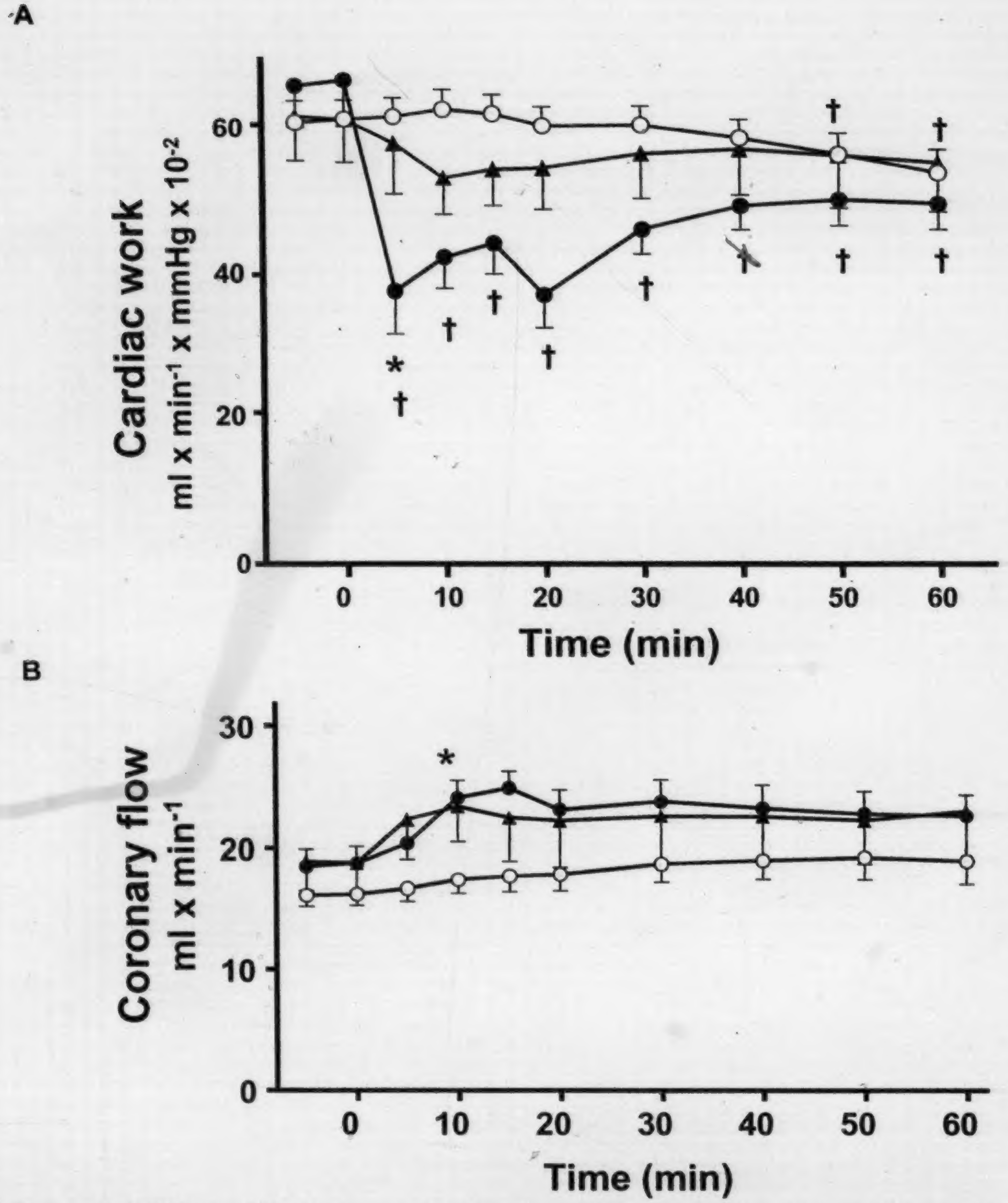

Figure 3.1 Effect of H_2O_2 in cardiac work and coronary flow in isolated working hearts. (A) Time course of the effects of 300 μM H_2O_2 on cardiac work in isolated rat hearts perfused for 60 min with Krebs-Henseleit buffer in absence (●, n=9) or in presence of 5 mM pyruvate (▲, n=5) as well as in Control conditions (○, n=7) For the pyruvate group there were no significant changes in cardiac work over the time. **(B)** Effects of 300 μM H_2O_2 on coronary flow in the presence or absence of 5 mM pyruvate. Values were significantly different from the –5 mark at and beyond 20, 10 and 5 min for the control, H_2O_2 and H_2O_2 + pyruvate groups, respectively. $^*p < 0.05$ vs. Control, †$p < 0.05$ vs. baseline (-5 min).

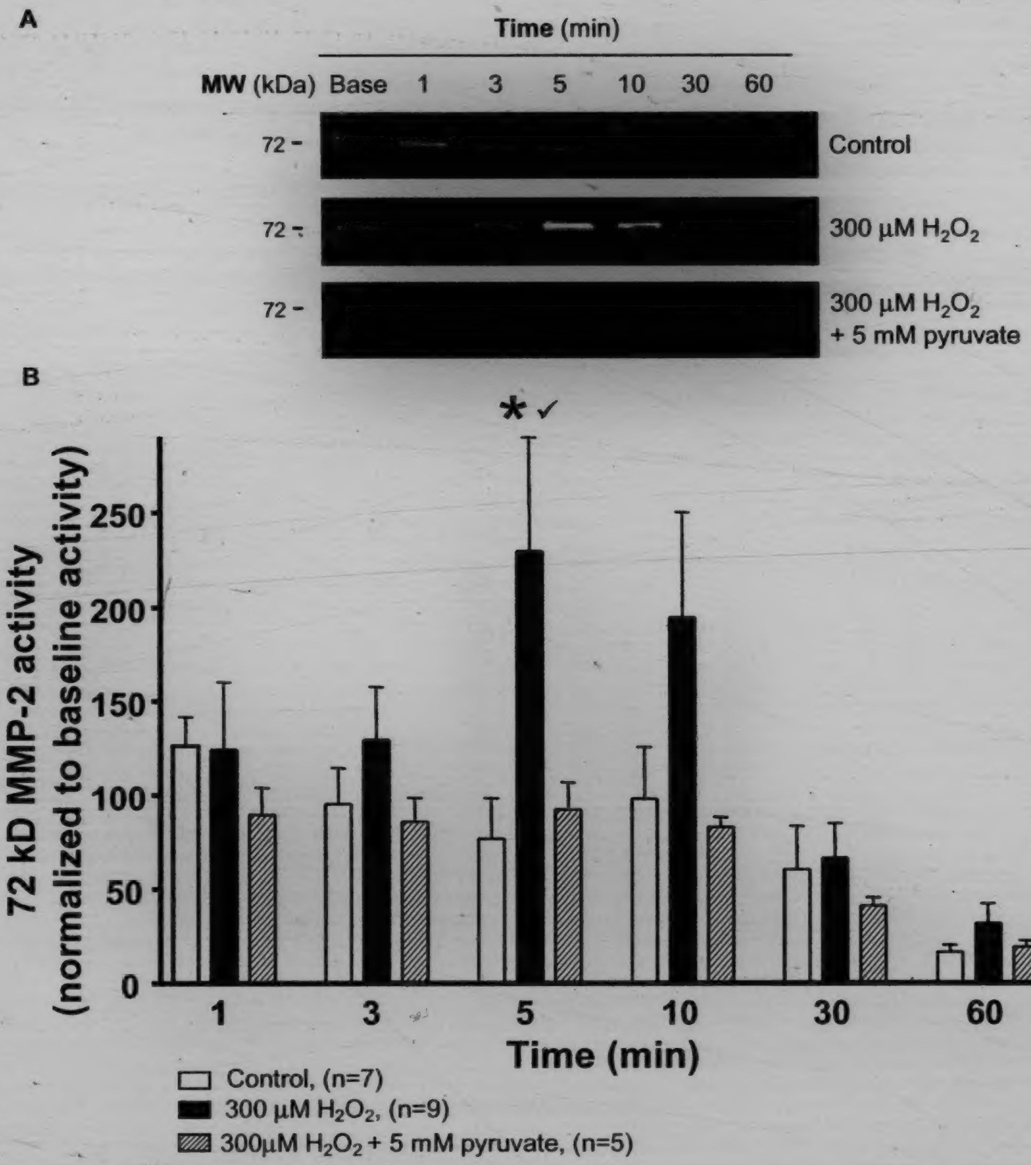

Figure 3.2 Time course 72 kDa MMP-2 release in the effluent during exposure to H_2O_2. (A) Representative zymograms of coronary effluents from Control, 300 µM H_2O_2, and 300 µM H_2O_2 + 5 mM pyruvate perfused hearts. **(B)** Densitometric analysis of the 72 kDa MMP-2 activity released in the effluent normalized to baseline (Base) values. *$p < 0.05$ vs. Control. ✓$p < 0.05$ vs. 300 µM H_2O_2 + 5 mM pyruvate.

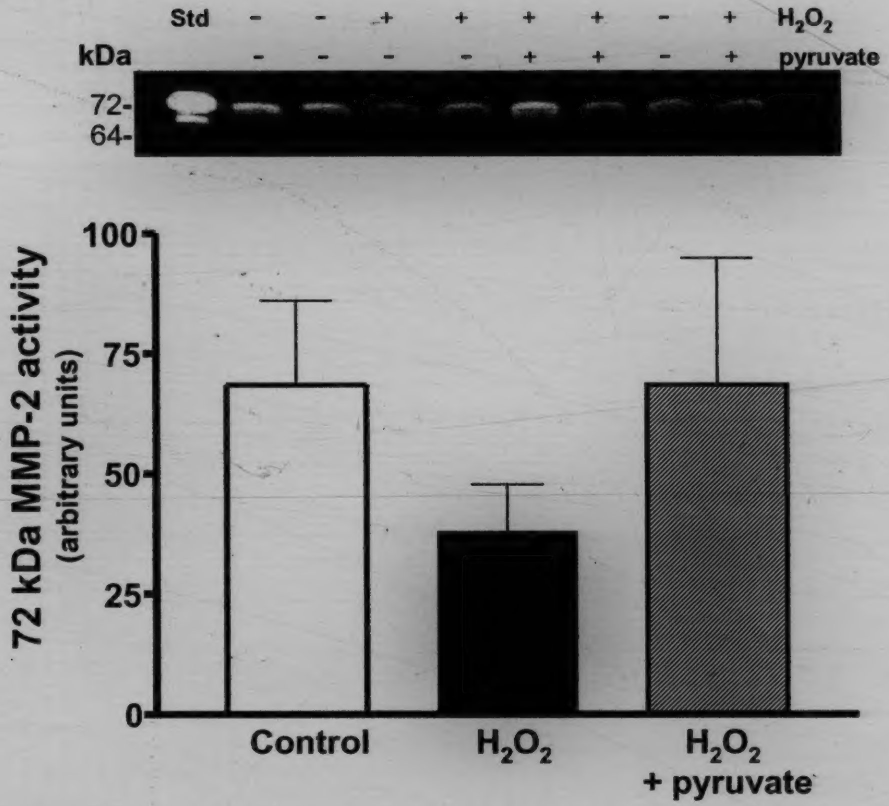

Figure 3.3 Effects of H_2O_2 on 72 kDa MMP-2 activity in heart homogenates. The upper panel shows a representative zymogram of homogenates prepared from perfused hearts exposed to H_2O_2 in presence or absence of 5 mM pyruvate, as well as from Control hearts. Hearts were frozen after 60 min of perfusion. Lower panel represents the densitometric analysis for the 72 kDa MMP-2 band. The open bar denotes Control hearts (n=7), filled bar represents hearts perfused for 60 min subjected to 300 μM H_2O_2 (n=9) and hashed bar depicts hearts subjected to H_2O_2 in presence of 5 mM pyruvate (n=5). Std: HT1080 conditioned cell media standard.

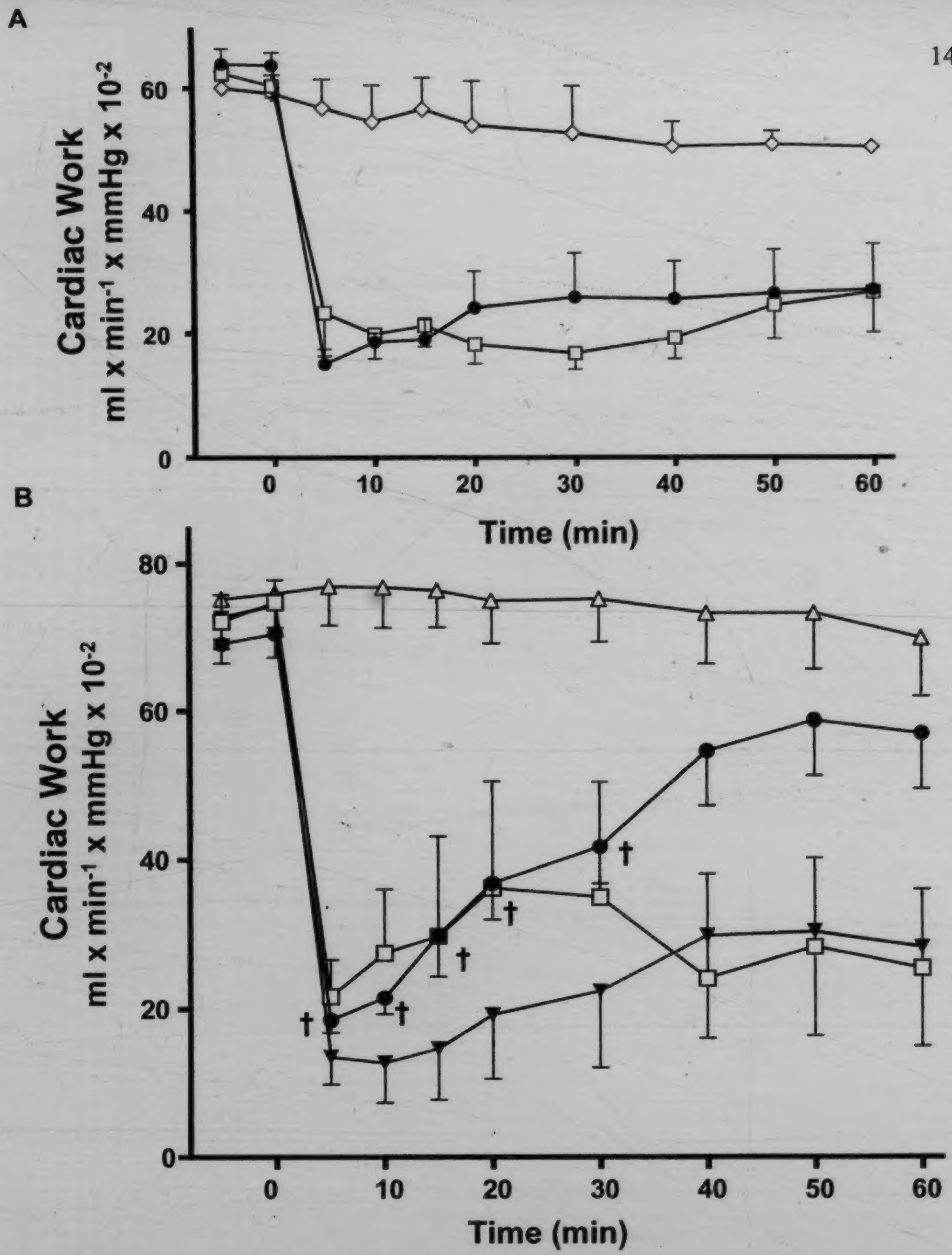

Figure 3.4 Effects of MMPs inhibitors on cardiac work in hearts exposed to H_2O_2. (A) Cardiac work in hearts treated with 300 μM H_2O_2 + 75 μM doxycycline (□, n=5) vs. hearts exposed to H_2O_2 alone (●, n=5). ◇ denotes hearts subjected to doxycycline in absence of H_2O_2 (n=3). Values were significantly different from the –5 mark for the H_2O_2, H_2O_2 + doxycycline groups. (B) Effect of Ro 31-9790 (□, n=5) and DMSO vehicle (▼, n=5) on cardiac work in hearts exposed to H_2O_2 in comparison to H_2O_2 alone (●, n=5). △ depicts hearts perfused with Ro 31-9790 alone, n=3. Values were significantly different from the –5 mark for the H_2O_2 + Ro 31-9790 and H_2O_2 + DMSO groups, respectively. †$p < 0.05$ vs. baseline (-5 min) in the same treatment group.

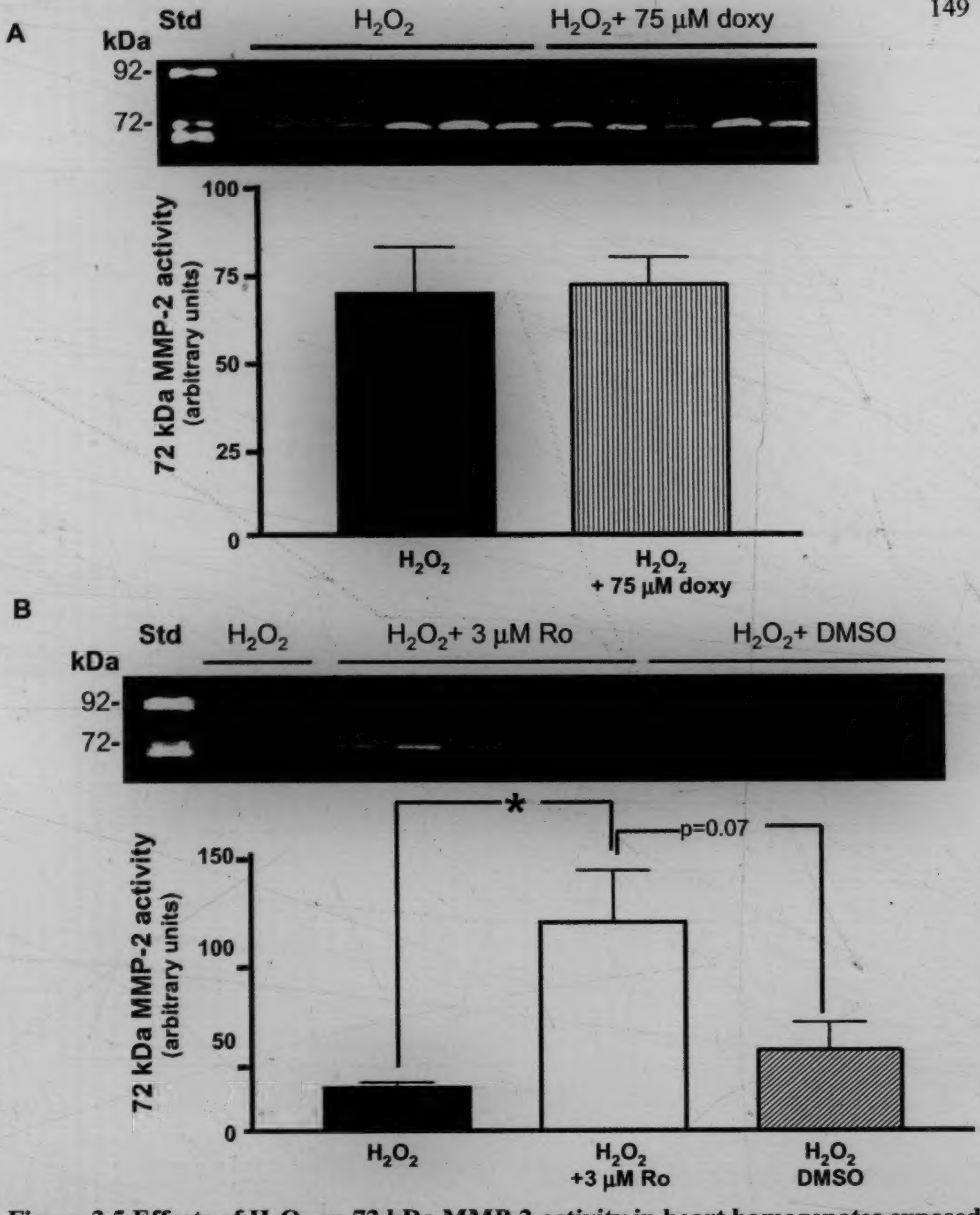

Figure 3.5 Effects of H_2O_2 on 72 kDa MMP-2 activity in heart homogenates exposed to H_2O_2 in presence of MMPs inhibitors. (A) Upper panel shows a representative zymogram of homogenates prepared from perfused hearts exposed to H_2O_2 in presence or absence of 75 µM doxy. The filled bar represents hearts perfused for 60 min subjected to 300 µM H_2O_2 (n=5) and the hashed bar denotes hearts exposed to H_2O_2 treated with 75 µM doxy (n=5). **(B)** Upper panel shows a representative zymogram of homogenates prepared from perfused hearts exposed to H_2O_2 in presence or absence of Ro or its vehicle. Lower panel depicts densitometric analysis of hearts exposed to H_2O_2 in presence of Ro or its vehicle (n=5/group). $^*p<0.05$ vs. H_2O_2.

Table 3.1 High-energy phosphates concentration in heart tissue after 60 min exposure to 300 μM H_2O_2[¶]

Group	ATP	ADP	AMP	AMP/ATP Ratio	PCr	Cr	PCr/Cr Ratio
Control (n=8)	19.9 ± 3.9	9.7 ± 1.7	3.7 ± 0.8	0.16 ± 0.04	61.9 ± 10.7	56.8 ± 10.7	1.1 ± 0.1
300 μM H_2O_2 (n=9)	24.0 ± 2.8	11.8 ± 3.6	2.3 ± 0.5	0.10 ± 0.02	91.0 ±10.4	55.9 ± 5.3	1.6 ± 0.1
300 μM H_2O_2 + 5 mM pyruvate (n=5)	27.3 ± 2.4	8.6 ± 0.6	2.0 ± 0.2	0.08 ± 0.01*	91.9 ± 10.8	53.2 ± 10.8	1.3 ± 0.2

[¶] Concentration of high-energy phosphates is given in μmol/g dry wet weight
*$p < 0.05$ vs. Control. *(Data collected with help of K. Strynadka)*.

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CHAPTER 4

DEGRADATION OF MYOSIN LIGHT CHAIN IN ISOLATED RAT HEARTS SUBJECTED TO ISCHEMIA–REPERFUSION INJURY: A NEW INTRACELLULAR TARGET FOR MATRIX METALLOPROTEINASE-2

This work was published: Sawicki G*, León H*, Sawicka J, Sariahmetoglu M, Schulze CJ, Scott PG, Szczesna-Cordary D, Schulz R. Degradation of myosin light chain in isolated rat hearts subjected to ischemia–reperfusion injury: A new intracellular target for matrix metalloproteinase-2. *Circulation.* 2005; 112:544-52. *Equal co-first authorship

4.1 INTRODUCTION

Ischemia-reperfusion (I/R) injury is a pathological condition which results from an acute increase in oxidative stress during reperfusion following ischemia which triggers a cascade of pathophysiological events including activation of MMPs.[1-4] The MMPs, in particular MMP-2, have been implicated in the pathogenesis of several other cardiovascular diseases including myocardial infarction,[5-7] heart failure,[8-10] pro-inflammatory cytokine-induced cardiac dysfunction,[11] reperfusion injury following heart transplant,[12] and cardiac dysfunction produced by endotoxemia.[13] MMPs activity is regulated at transcriptional and post-transcriptional levels, including their inhibition by endogenous TIMPs.

Although MMPs are best known for their actions in remodeling the extracellular matrix, we recently showed that the acute contractile dysfunction in myocardial I/R injury is caused in part by MMP-2, an abundant MMP expressed in several cells including cardiac myocytes, which acts intracellularly by degrading troponin I.[2] MMP-2 activity in I/R injury is stimulated by $ONOO^-$[14,15] generated in early reperfusion,[16] the peak biosynthesis of which precedes MMP-2 activation.[1] Indeed, direct infusion of $ONOO^-$ into isolated hearts activated MMP-2 prior to the onset of contractile failure, the latter which was prevented by MMP inhibition.[17] A net positive proteolytic balance also ensues as a result of I/R due to loss of TIMP-4 from the myocardium.[3] However, whether MMP-2 has other intracellular targets in the I/R heart in addition to troponin I is unknown.

In order to address this, we subjected isolated rat hearts to I/R injury and used a combined pharmacological and functional proteomics approach to analyze protein changes. We discovered that myosin light chain 1 (MLC1) is another proteolytic target of MMP-2 in this setting.

4.2 METHODS

This investigation conforms to the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care.

4.2.1 Heart perfusion and ischemia-reperfusion protocol

Male Sprague-Dawley rats (250-300g) were used for the experiments. Hearts were excised from anesthetised rats and perfused via the aorta at constant pressure (60 mmHg) with Krebs-Henseleit buffer at 37°C as previously described.[1,18] Flow rate, heart rate and left ventricular pressure were monitored. Left ventricular developed pressure was calculated as the difference between systolic and diastolic pressures of the left ventricular pressure trace. The rate-pressure product was calculated as the product of the spontaneous heart rate and left ventricular developed pressure. Using our perfusion protocol, hearts maintained a steady state of coronary flow, heart rate and left ventricular developed pressure for at least 80 min after stabilization.[18]

Following 25 min of aerobic perfusion, hearts were subjected to 20 min global, no-flow ischemia induced by clamping the aortic inflow line. This was followed by 30 min of aerobic reperfusion by reopening the clamp. Control hearts were perfused aerobically for 75 min. The hearts (n=6 per group) were freeze-clamped and immersed in liquid nitrogen at the end of the protocol.

In some experiments, after 15 min of aerobic perfusion, inhibitors of MMPs activity, o-phenanthroline (Phen, 100 μM) or doxycycline (Doxy, 100 μM)[1,2] were infused into hearts for the last 10 minutes of aerobic perfusion and for the first 10 minutes of reperfusion.

4.2.2 Preparation of heart extracts

Protein samples for 2-D electrophoresis were prepared at room temperature by mixing frozen (-80°C), powdered heart tissue (40-60 mg wet weight) with 200 μL of rehydration buffer (8 M urea, 4% CHAPS, 10 mM DTT, 0.2% Bio-Lytes 3/10 [BioRad]) at room temperature. Samples were sonicated twice for 5 seconds and centrifuged for 10 min at 10,000 g at room temperature to remove any insoluble particles. Protein content of the heart extract in rehydration buffer was measured using the BioRad protein assay. For other biochemical studies frozen heart tissue powder was homogenized on ice in 50 mM Tris-HCl (pH 7.4) containing 3.1 mM sucrose, 1 mM DTT, 10 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, 2 μg/mL aprotinin, and 0.1% Triton X-100. Homogenates were centrifuged at 10,000 g at 4°C for 10 min and the supernatant was collected and stored at -80°C until use.

4.2.3 Two-dimensional polyacrylamide gel electrophoresis

100 or 400 μg of heart extract protein was applied to 11 cm immobilized pH gradient strips (IPG, BioRad), and equilibrated for 16-18 h at 20°C in rehydration buffer. The 11 cm IPG strips have linear pH gradients of either 3-10, 3-6 or 5-8. For isoelectrofocussing, the BioRad Protean isoelectrofocussing cell was used with the

conditions described previously.[19] Second dimensional electrophoresis was carried out using Criterion precast gradient gels, 8-16% or 4-12% acrylamide (BioRad). To minimize variations in resolving proteins during the second dimension run, 12 gels were run simultaneously using a Criterion Dodeca Cell (BioRad). After separation, proteins were detected using Coomassie Brilliant Blue R-250 (BioRad). Because we could not run more than twelve 2-D gels simultaneously (3 gels per group: control, I/R, I/R + doxycycline and I/R + phenanthroline) we combined the extracts from 2 hearts in the same group and applied them to one gel (200 μg protein from each sample). For statistical analysis the n number that we used was 3. All the gels were stained in the same bath. Equivalent protein loading by determination of actin spot intensity, as well as inter-gel reproducibility of spot intensity was confirmed (see below).

Developed gels were scanned using a calibrated GS-800 densitometer (BioRad). Quantitative analysis of spot intensity from 2-D electrophoresis was measured using PDQuest 7.1 software (BioRad) and intensities of the separate bands from SDS-PAGE were analyzed and expressed in arbitrary units using Quantity One 4.4 measurement software (BioRad). The protein spot sensitivity threshold we used to determine significant changes in protein spot size and density is based on four parameters: minimum peak value sensitivity, smallest spot area, largest spot area, and a noise filter level. Only protein spots with relative intensity between 10-100 arbitrary units were considered for analysis. Using these criteria for protein resolution and staining, we were able to obtain high reproducibility to analyze both a single protein from the same sample run in different gels[19] and for a specific protein spot from different heart samples. We used a pharmaco-proteomics approach in which we only evaluated protein spots which changed from aerobic control samples as a result of I/R injury and were normalized in hearts subjected to I/R in the presence of both doxycycline or phenathroline.

4.2.3.1 Evaluation of contractile protein solubilization

In order to evaluate the efficiency of the solubilization of contractile proteins in the extracts using rehydration buffer, 50 μg of protein from Aerobic Control heart extracts (n=3) were electrophoresed using a 15% SDS-polyacrylamide gel according to Laemmli[20] and visualized with Coomassie Brilliant Blue R-250. Comparison of the obtained protein pattern with the known protein band using appropriate standards from pig cardiac muscle suggests that the use of rehydration buffer for protein solubilization efficiently extracted contractile proteins from the myocardium (Figure 4.1A).

4.2.3.2 Evaluation of protein loading and 2-D electrophoresis reproducibility

In order to verify the reproducibility of protein loading of individual heart extracts onto the IPG strips, the level of actin in these extracts from each of the hearts from all four experimental groups was evaluated by 2-D electrophoresis. 100 μg total protein from each heart extract was applied onto an 11 cm strip (pH range 3-6). The first and second dimensions were run as described above. The actin spot was identified by mass spectrometry. The quantitative analysis of actin spot intensities from the 2-D gels showed no differences in the group averaged actin spot intensities between Aerobic control hearts and those subjected to I/R with or without MMP inhibitors (Figure 4.1B).

4.2.3.3 Reproducibility of protein spot quantification between 2-D gels

Protein extracts (n=3) from the combined pairs of aerobic control hearts (total n=6 hearts) were used to assess the reproducibility of the 2D protein spot pattern obtained from several hearts within the same experimental group. We correlated the intensity of approximately 40-50 protein spots per gel which fit our inclusion criteria for quantitative evaluation of 2-D gels (spot intensity between 10-100 arbitrary units) using PD Quest. The results show significant and high positive correlation (from 0.764 to 0.828) of protein spot intensity when comparing one gel to another (Figure 4.2).

4.2.4 Mass spectrometry

Protein spots that demonstrated statistically significant changes in spot size and density parameters described above were manually excised from the 2-D gel. Subsequently, protein sequence data for their identification was obtained by in-gel digestion with trypsin and LC/MS/MS. The Mowse scoring algorithm[21] was used for justification of protein identification.

Intact protein mass was measured by mass spectrometry. The excised gel fragment containing the protein spot was first de-stained in 200 μL of 50% acetonitrile with 50 mM ammonium bicarbonate at 37°C for 30 minutes, next the gel was washed twice with water. The protein extraction was performed overnight at room temperature with 50 μL of a mixture of formic acid, water and isopropanol (1:3:2, v:v). The resulting solution was then subjected to MS. For electrospray Q-TOF (quadruple time of flight) analysis 1 μL of the solution was used. LC/MS was performed on a CapLC HPLC (Waters, USA) coupled with a Q-TOF-2 mass spectrometer (Micromass, UK).

4.2.5 *In vitro* degradation of myosin light chain 1

Because a commercial preparation of rat MLC1 is not available we used rabbit MLC1 (gift from Dr. P. Fajer, Florida State University)[22] for *in vitro* degradation of MLC1 by MMP-2 or MMP-9. Comparison of the primary structures of rat and rabbit MLC1 shows 74% identity for all 192 amino acids. Purified rabbit MLC1 (10 μg) was incubated with human recombinant MMP-2 or MMP-9 (0.30 μg MMP-2 or 0.38 μg MMP-9, Oncogene)[2] in 50 mM Tris-HCl buffer (5 mM $CaCl_2$, 150 mM NaCl, total volume 60 μL) at 37°C for either 20 or 60 min. The reaction mixtures were analyzed by 15% SDS-PAGE under reducing conditions and visualized by the Coomassie brilliant G-250 staining method. Parallel experiments were performed using the same conditions described above but MMP-2 was added at the end of the incubation period as a negative control. The molecular weight of MLC1 degradation products was calculated using Quantity One 4.4 software (BioRad).

4.2.6 Myosin light chain 1 sequence analysis

Using the LALIGN peptide comparison program (*www.ch.embnet.org/software/LALIGN_form.html*), the primary sequence of rat ventricular myosin light chain 1 (MLC1) was compared to known MMP-2 cleavage recognition sites.[23-25] Sites with homology greater than 60% were considered in the analysis. We also compared the homology of rat MLC1 with rabbit MLC1 using the same program.

Secondary structure and a 3-D model of rat ventricular MLC1 (Swiss-Model Repository code P16409C0001.pdb) was created based on X-ray crystal structure of the chick gizzard smooth muscle form of this protein (RCSB Protein Data Bank accession code 1BR1.pdb).

4.2.7 Immunogold electron microscopy

Immunogold labeling of heart tissue was performed as previously described.[2] To determine the specificity of the anti–MMP-2 antibody, it was incubated with recombinant MMP-2 for 30 minutes at 37°C in a 1:5 molar ratio in high stringency RIPA buffer (50 mM Tris-HCl [pH 8.0] 150 mM sodium chloride, 1% NP-40, 0.1% SDS, and 0.5% sodium deoxycholate) before routine staining for MMP-2. Sections were examined using a Hitachi H-7000 transmission electron microscope at 75 kV.

4.2.8 Thick myofilament preparation

Cardiac myosin thick filaments were isolated from frozen rat ventricle tissue powder according to Svensson *et al.*[26] Briefly, the powder was dissolved and homogenized in Guba-Straub buffer (300 mM NaCl, 100 mM NaH_2PO_4, 50 mM Na_2HPO_4, 10 mM $Na_4P_2O_7$, pH to 6.5, 1 mM $MgCl_2$, 10 mM EDTA, 1 mM DTT, 0.1% NaN_3, and leupeptin 10 μg/mL), incubated on ice for 20 min while stirring and then centrifuged at 4°C for 10 min at 30,000*g*. The supernatant was diluted with 12 vol of 1 mM EDTA, pH 7.0, stirred for 30 min and then left to stand for 30 min. The sample was centrifuged again as described. The pellet was then re-suspended in low salt buffer (1 mM $MgCl_2$ and 1 mM EGTA, pH 7.0 with freshly added 1 mM ATP and 1 mM DTT) and centrifuged as before. The final pellet was re-dissolved in 10 mM MOPS (pH 7.0), 0.4 M KCl and 1 mM DTT and stored in 50% glycerol at -20° C.

4.2.9 Measurement of MMP-2 by zymography

Gelatin zymography was performed as described[7]. Briefly, thick filament preparations were applied to an 8% polyacrylamide gel copolymerized with 2 mg/mL gelatin. After electrophoresis, gels were rinsed three times for 20 min each in 2.5% Triton X-100 in order to remove SDS. Then the gels were washed twice in incubation buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 150 mM NaCl and 0.05% NaN_3) for 20 min each at room temperature and then incubated in incubation buffer at 37°C. The gels were stained in 2% Coomassie Brilliant blue G, 25% methanol, 10% acetic acid for 2 hrs and then destained for 1 hr in 2% methanol/4% acetic acid.

4.2.10 Immunoprecipitation

300 μg of heart extract proteins were incubated with 12 μg of rabbit anti-MMP-2, fragment 1 IgG[1] in a total volume of 50 μL RIPA buffer (50 mM Tris pH 8.0 with 150 mM $NaCl_2$, 1% sodium deoxycholate and 0.1% SDS) overnight at 4°C. This buffer was chosen because of its known high stringency to avoid unspecific binding. As a negative control, unrelated IgG was used instead of anti MMP-2 IgG. 100 μl of slurry of protein A–Sepharose beads was added and the mixture was incubated overnight at 4°C. The mixture was washed 3 times with 0.5 mL of RIPA buffer at 4°C and 20 μL of sample buffer was added to elute the samples from the beads as described.[20] The immunoprecipitates were analyzed by Western blot with anti MLC1 IgG.

4.2.11 Western blot

MLC1 content in myocardium was determined by Western blot. 20 μg protein from each heart extract was separated using 15% SDS-PAGE[20] and transferred to a polyvinylidene difluoride membrane (Bio-Rad). MLC1 was identified using a monoclonal anti-human MLC1 antibody (Accurate Chemical and Scientific Corporation). Band densities were measured using GS-800 calibrated densitometer and Quantity One software.

4.2.12 Statistical analysis

Data are shown as mean ± SEM. Functional data and the *in vitro* degradation of MLC1 data were analyzed using ANOVA with the Tukey-Kramer multiple comparison test. Analysis of the protein spots in 2-D electrophoresis experiments was performed using the Kruskal-Wallis test for non-parametric values. A value of $p<0.05$ was considered statistically significant.

4.3 RESULTS

4.3.1 Functional protection by MMPs inhibition during I/R injury

The functional recovery of the hearts after 30 min of reperfusion following 20 min ischemia was significantly reduced in the I/R group compared with hearts aerobically perfused for 75 min (to 26±7% of aerobic heart function, n=6, $p<0.001$). Inhibitors of MMPs, doxycycline or o-phenanthroline, significantly improved the recovery of mechanical function after 30 min reperfusion (Figure 4.3).

4.3.2 Analysis of proteins in heart extracts separated by 2-D electrophoresis

For the separation of myocardial homogenate proteins by 2-D electrophoresis we used for the first dimension wide range (pH 3-10) IPG strips, and for the second dimension we used gradient gels (4 to 12% polyacrylamide). The 2-D gels showed that the majority of proteins were localized near the centre of the gel, in the mid pH range and toward the lower molecular weight range (data not shown). Therefore, we repeated 2-D electrophoresis of the heart extracts using narrow range strips (pH 5-8) for the first dimension and 8 to 16% gradient gels in the second dimension (Figure 4.4A).

Our approach was to identify those proteins whose spot intensity was affected by I/R and which was then normalized in I/R hearts treated with both inhibitors of MMPs. Using our criteria for protein spot size and density, we found only three protein spots whose spot intensity changes fitted these criteria (proteins 1, 2 and 3, Figure 4.4). Because protein 1 was found in very close proximity to protein 2 in the horizontal axis suggesting that they represent the same species but different post-translational modifications, we analyzed their intensities together for quantitative analysis but not for identification. The densitometric analysis of the three protein spots showed that the levels of both proteins 1+2 and protein 3 significantly increased upon I/R injury. MMPs inhibitors, o-phenanthroline or doxycycline, normalized the values of these proteins to that observed with control aerobic perfusion (Figure 4.5).

Mass spectrometry analysis of each of the excised, solubilized and trypsin digested spots identified that protein spots 1 and 2 as well as spot 3 are fragments of myosin light chain 1 (MLC1). Spot 4 was identified as intact MLC1 (Table 4.1). Details of the protein spot identification by mass spectrometry are shown in Table 4.1.

4.3.3 Analysis of MLC1 sequence for MMP-2 cleavage recognition sequences

Comparison of the amino acid sequence of rat MLC1 with various MMP-2 cleavage recognition sequences[19-21] revealed two sites of interest at the C-terminus (Figure 4.6A). One site between amino acids 171 and 178 shows 75% homology with the 8 amino acid sequence recognized by MMP-2, and a second site between amino acids 187 and 191 showed 60% identity with overlap of 5 amino acids.

We then compared the peptide sequences obtained by MS/MS analysis from all 4 trypsin-digested protein spots (Figure 4.6B). Protein spots 1 and 2 represent MLC1 that is shortened by cleavage between lysine 175 and lysine 194. The shortest protein, spot #3, represents MLC1 missing a peptide produced by cleavage between arginine 167 and lysine 175. Amino acid sequences for all four MLC1 forms are identical from the N-terminus to arginine 167 (data not shown).

It is known that the α-helix secondary structure protects proteins from proteolytic degradation. Comparative analysis of the secondary structure of native rat MLC1 (Figure 4.6C) reveals that the predicted cleavage site between 171 and 178 is almost entirely located within this α-helix region whereas the cleavage site between 187 and 191 amino acid has both extended β strand and α-helix structure.

In order to examine the spatial location of the hypothetical cleavage sites in MLC1 we created a 3-D structure for this protein. Figure 4.7 shows that the two theoretical cleavage sites in MLC1 are located on the surface of the molecule, making them more readily accessible for proteolysis by MMP-2.

4.3.4 Analysis of undigested MLC1 spots for identification of possible cleavage sites for MMP-2

Measurement of the molecular masses of undigested protein spots 1, 2 and 4 by mass spectrometry confirmed that spot 4 represents intact MLC1 with mass 22323.6±3.9 Da and protein spots 1 and 2 represent truncated forms of MLC1 with masses of 21193.2±4.7 Da and 21193.6±7.2 Da, respectively (Figure 4.8). The level of protein in spot 3 was below the detection limit for this analysis. The result from peptide analysis (Figure 4.8) strongly suggests that a short peptide (no larger than 24 amino acids) is absent from the C-terminal part of MLC1 in the truncated forms. Mass spectrometry shows that both truncated forms (spots 1 and 2) are smaller by approximately 1130 Da, corresponding to 10 amino acids (average mass of amino acid in MLC1 is 110 Da). The loss of a 10 amino acid peptide from the C-terminus of MLC1 suggests that the peptide bond between tyrosine (Y)189 and glutamate (E)190 is indeed the true cleavage site (Figure 4.9). This result is consistent with the theoretical prediction of possible MMP-2 cleavage sites within MLC1 (Figure 4.6A).

4.3.5 *In vitro* degradation of MLC1 by MMP-2

In order to examine the susceptibility of purified MCL1 to proteolytic degradation by MMP-2, MMP-2 was incubated with rabbit MLC1. The homology around the hypothetical cleavage sites of rabbit and rat MLC1 are almost identical. Degradation products of MLC1 were detected within 20 min incubation at 37°C with MMP-2. A major degradation product of 19.5 kDa (Figure 4.10A) was observed. After 60 min incubation (Figure 4.10B) the degradation of MLC1 was enhanced and two major degradation products of 18.5 and 14.7 kDa were seen. We also determined the susceptibility of MLC1 to degradation by MMP-9 under the same conditions. We did not observe any degradation of MLC1 by MMP-9 (60 min incubation at 37°C, data not shown).

4.3.6 Localization of MMP-2 within the sarcomeres and its association with MLC1

We confirmed using immunogold electron microscopy of rat hearts subjected to I/R using anti-MMP-2 that MMP-2 is localized in the sarcomere.[2] Positive immunogold staining was found mostly in the region corresponding to the A band and to a smaller extent in the region corresponding to the I band and the Z disc (Figure 4.11). A sparse amount of positive staining for MMP-2 was observed in the H band and M line. Control experiments (using anti-MMP-2 IgG pre-absorbed with MMP-2) were devoid of positive MMP-2 staining. This distribution pattern of MMP-2 staining within the sarcomere is consistent with the distribution and location of myosin light chain.

In addition, gelatin zymography of purified thick filament preparations from I/R hearts showed gelatinolytic activity corresponding to both 72 and 62 kDa forms of MMP-2 (Figure 4.12A). Immunoprecipitation of MLC1 with anti-MMP-2 IgG in rat heart homogenates followed by Western blot analysis for MLC1 (Figure 4.12B) reveals the association of intact MLC1 with MMP-2 in aerobic control hearts and the degradation product of MLC1 with MMP-2 in hearts subjected to I/R.

4.4 DISCUSSION

In this study we demonstrated that MLC1 is a novel target of MMP-2 action in the setting of acute myocardial ischemia-reperfusion injury in the isolated rat heart. 2-D electrophoresis shows that MLC1 exists in the heart in at least 4 different molecular forms which differ in molecular weight, post-translational modifications and concentration. The administration of MMP inhibitors not only improves the functional recovery after I/R but also prevents the degradation of MLC-1. Proteolytic degradation of MLC1 was proposed in earlier studies of I/R injury to rat hearts, however, the protease responsible for this was not identified.[27] MLC1 proteolysis could lead to contractile dysfunction due to a decrease in the stability of the myosin neck region which may affect the kinetics of cross-bridge cycling.[26] MLC1 degradation products have been observed in the heart following myocardial infarct in human[28] and dogs,[29] as well as in heart failure,[30] which may in part explain the contractile dysfunction associated with these diseases.

Since the discovery of MMPs in 1962 they have been considered as extracellular matrix proteinases responsible for remodeling the matrix and degradation of its components. Discovery of an intracellular function of MMP-2 to proteolyze troponin I during ischemia-reperfusion injury[2] challenged this notion. MMP-1, -8 and -9 were shown to be activated by $ONOO^-$ by S-glutathiolation of a cysteine residue in the autoinhibitory propeptide domain. Although this has not yet been explicitly shown for MMP-2, the autoinhibitory PRCGVPD domain is highly conserved across all MMPs. As the generation of $ONOO^-$ in the reperfused heart peaks within the first minute of reperfusion[16] and MMP-2 activity peaked within the first 2-5 min of reperfusion[1] we speculate that $ONOO^-$-induced S-glutathiolation of MMP-2 would result in active MMP-2 in the intracellular compartment. Intracellular activation of MMP-2 via proteolysis has also been documented.[31] Further studies are needed to test these hypotheses.

Our results suggest that MMP-2 plays an important role in the degradation of MLC1. This is based on the fact that MMP-2 was associated with thick myofilaments as observed by four different methods. Immunogold electron microscopy shows that MMP-2 is preferentially localized to the A and I band regions, but not the H band (which lacks both MLC and troponin). The A band contains the heads of the myosin complex which include both MLC1 and MLC2 in association with the troponin complex.[32-34] Furthermore, we also see that MMP-2 is localized in the I band which lacks myosin but contains the troponin complex, including troponin I which was previously shown to be cleaved by MMP-2.[2] Using immunogold electron microscopy we also observed the presence and a similar distribution of MMP-2 in the sarcomeres of aerobically perfused control hearts (data not shown). It is possible that this MMP-2 is in its latent form. Preparations of thick myofilaments show gelatinolytic activity corresponding to MMP-2 and MLC1 can be immunoprecipitated from heart homogenates with anti-MMP-2. Finally, MLC1 proves to be an excellent *in vitro* substrate for MMP-2, but not for a related gelatinase, MMP-9.

In the present study we observed degradation products of MLC1 in heart tissue with a shorter period of ischemia (20 min) than that observed by Van Eyk *et al.*[27] They found degradation products of MLC1 by Western blot in isolated myofibrils and in the effluents of rat hearts subjected to a longer period of ischemia (60 min) alone or 60 min of ischemia followed by 45 min reperfusion in Krebs-Henseleit buffer containing 1.15 mM free Ca^{2+}. It is well known that the susceptibility of the heart to I/R injury increases with higher free Ca^{2+} concentration in the perfusate.[35] The condition of 2.5 mM free Ca^{2+} used in the present study accounts for the more rapid development of injury seen only after 20 min of ischemia and 30 min reperfusion. Our results suggest that the degradation of MLC1 during ischemia occurs earlier than what was previously thought, with potential pathophysiological implications in the setting of myocardial stunning injury. In addition, the differences between the two studies may be explained in that we used 2-D gel electrophoresis and mass spectrometry which detects potential post-translational modifications or protein degradation products with higher sensitivity than the methods previously employed.[27] On the other hand, it is plausible that according to the severity of the ischemic insult, a number of mechanisms can be either triggered or accentuated leading to partial degradation of the contractile machinery as seen in the present study. A limitation of the present study is that we did not investigate the correlation between duration of ischemia and degradation of MLC1 in hearts, which we plan to do in future studies.

MLC1 is one of the sarcomeric proteins which plays an important role in cardiac muscle contraction. Any alterations in its structure could severely affect the contractile performance of the heart. Our results show that one of the actions of MMP-2 is to remove the C-terminal α-helix of MLC1. The relatively exposed locations of both α-helices at the surface and near the end of this protein which have amino acid sequences resembling known substrates for MMP-2 would probably facilitate cleavage of MLC1 within the open cleft at the active site of MMP-2.

Other proteases such as the caspases are known to proteolyze components of the thick and thin myofilaments.[36,37] Calpains may be involved in sarcomeric protein degradation following ischemic episodes more severe than that observed in our model of stunning injury.[38] The acute event of MMP activation and proteolysis of susceptible targets such as troponin I[2] or MLC1 may also trigger inflammatory signaling cascades which exacerbate heart function and promote myocyte apoptosis several hours after reperfusion.[39,40]

Various lines of evidence show that proteolysis of cytoskeletal proteins such as α-actinin,[27,41] spectrin,[41] desmin[41,42]as well as sarcomeric proteins other then MLC1 such as troponin I,[2,27,43,44] troponin C,[44] and actin[45] in the cardiac myocyte contribute to the development of I/R injury. Our previous finding of troponin I cleavage by MMP-2,[2] in addition to our present results with MLC1, suggest that MMP-2 plays an important role in the pathogenesis of acute I/R injury. Although these results point to, but do not unequivocally prove an intracellular action of MMP-2 in the cardiac myocyte, they provide a molecular basis for inhibition of MMPs as a means to protect the heart from ischemia-reperfusion injury.

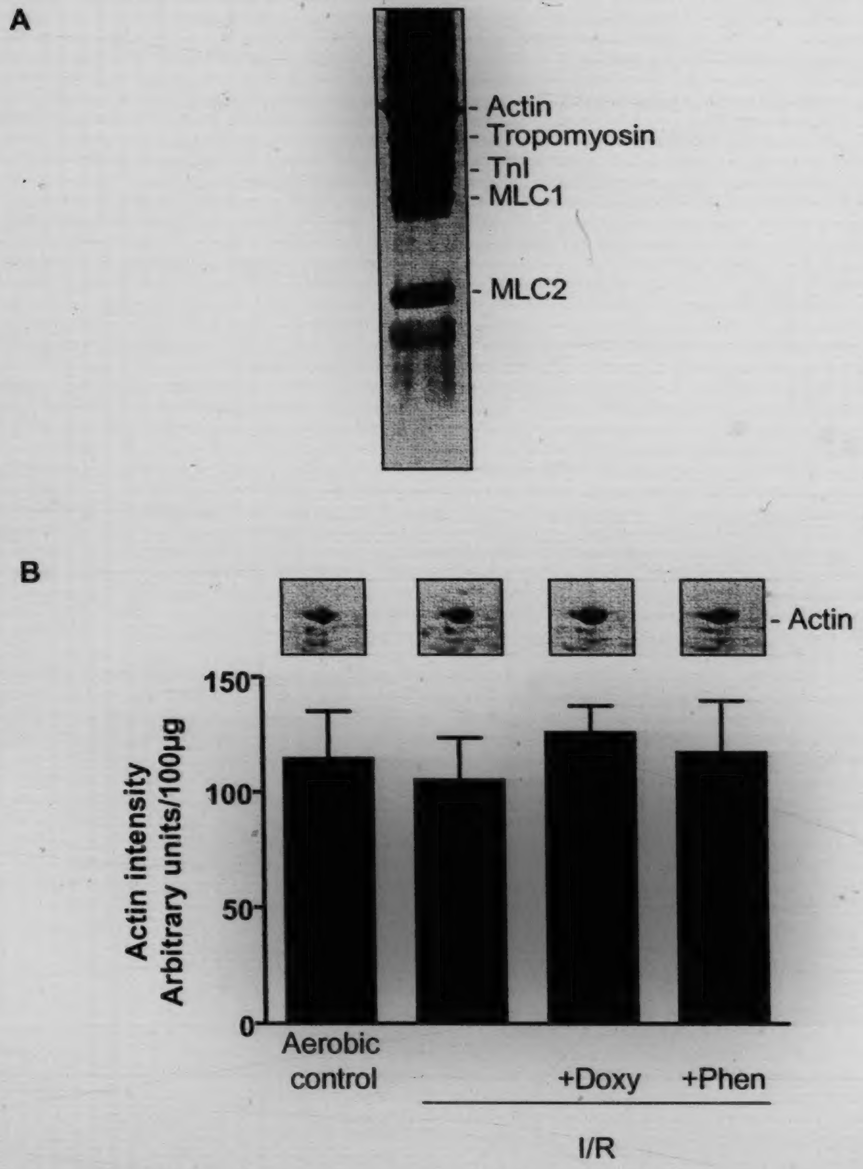

Figure 4.1 Contractile protein solubilization efficiency and reproducibility of protein loading in 2-D electrophoresis. (A) 15% SDS gel electrophoresis showing extraction of myocardial proteins in the rehydration buffer used for 2-D electrophoresis. (B) Densitometric analysis of actin spot intensities from heart extracts separated by 2-D gel electrophoresis. IPG strips with 3-6 pH gradient for the first dimension and 8-16% polyacrylamide gel gradient for the second dimension were used. 100 of µg total proteins were loaded per gel. The inserts show representative actin spots within the 2-D gel from each group. The border of the actin spots (dark line around the spot) was determined by using the automatic spot contour tool provided by PDQuest. The lower panel shows the quantitative analysis of actin spot intensities. *(2-D gel electrophoresis done with help from G. Sawicki).*

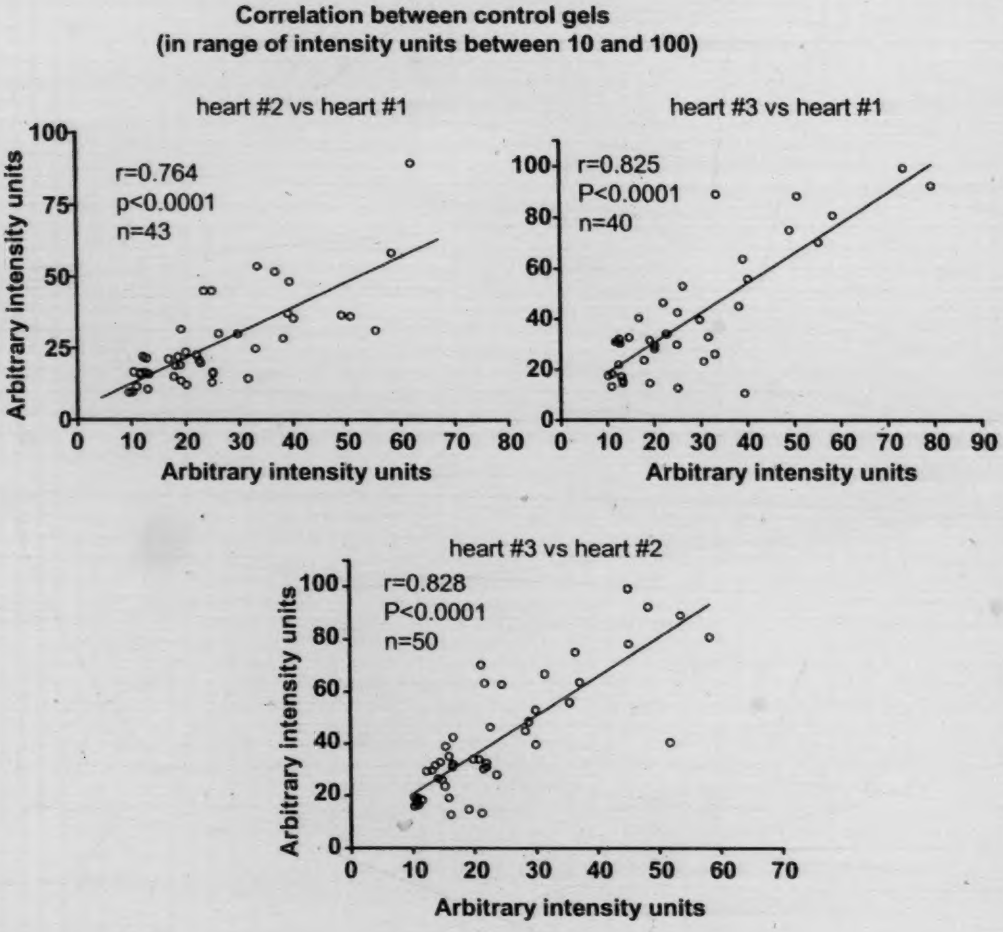

Figure 4.2 Reproducibility of protein spot quantification between gels. Correlation of spot intensities from 2-D gel electrophoretic analysis of Aerobic control hearts extracts. Each graph represents the comparison of spot intensities of 40-50 protein spots meeting our inclusion criteria as analyzed between two different gels within the same experimental group.

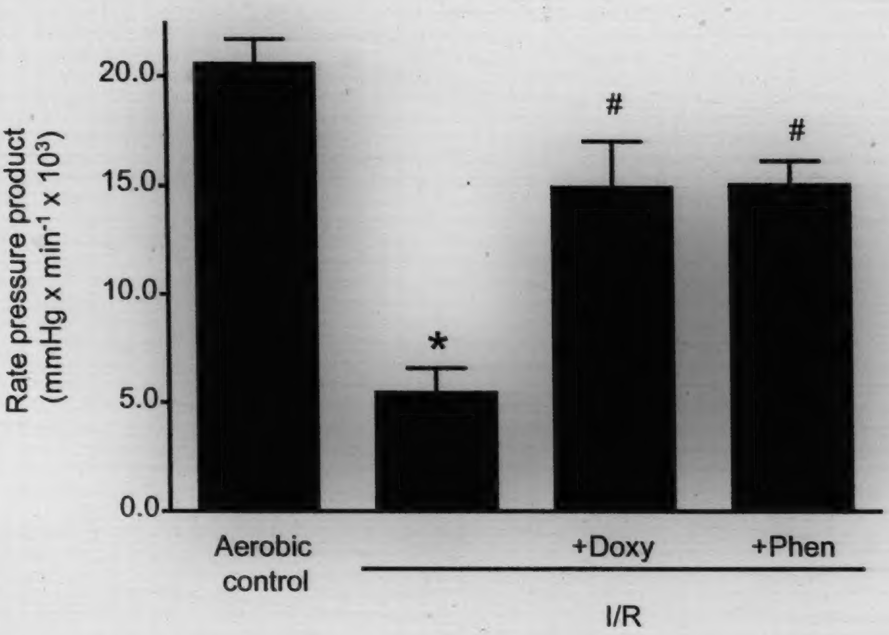

Figure 4.3 Effect of MMPs inhibitors on cardiac mechanical function (rate-pressure product) in isolated perfused rat hearts. Bar graph represents cardiac mechanical function from Aerobic control hearts and those subjected to 20 min ischemia and 30 min reperfusion (I/R) in the presence or absence of doxycycline (+Doxy) or phenanthroline (+Phen). $^{*}p<0.001$ vs. Aerobic control, $^{\#}p<0.001$ vs. I/R alone, n=6. *(Perfusions done by J. Sawicka).*

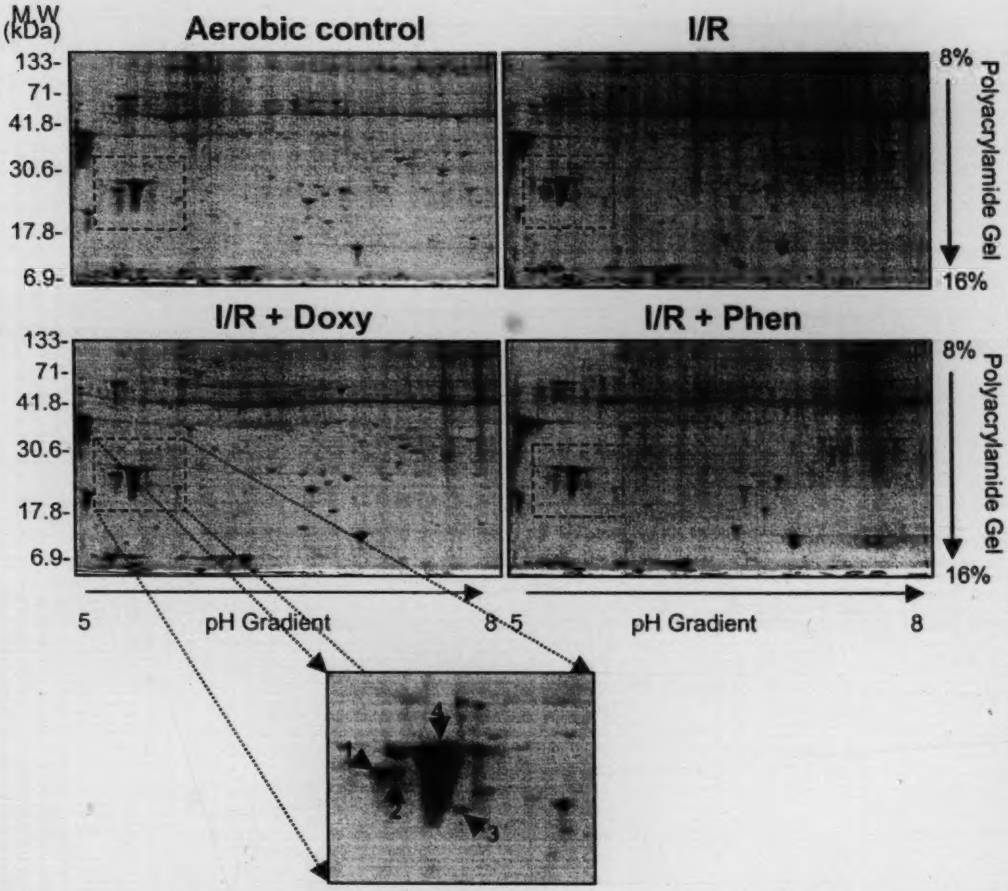
M.W (kDa)
Aerobic control
I/R
133-
71-
41.8-
30.6-
17.8-
6.9-
8%
Polyacrylamide Gel
16%
I/R + Doxy
I/R + Phen
5
pH Gradient
8
5
pH Gradient
8
1
2
3
4

Figure 4.4 Representative 2-D electrophoresis of heart homogenates from Aerobic control, I/R, I/R+Doxy and I/R+Phen groups using a narrow range pH strip (5-8) and 8-16% polyacrylamide gradient gel. 400 μg total proteins were loaded per gel. A representative gel from each group is shown. The square indicates the region of the gel where protein levels were affected by I/R in comparison to Aerobic control and were then normalized by doxycycline (I/R+Doxy) or phenanthroline (I/R+Phen). Representative enlargement of the gel showing the marked region is shown. *(Experiments done with help from G. Sawicki).*

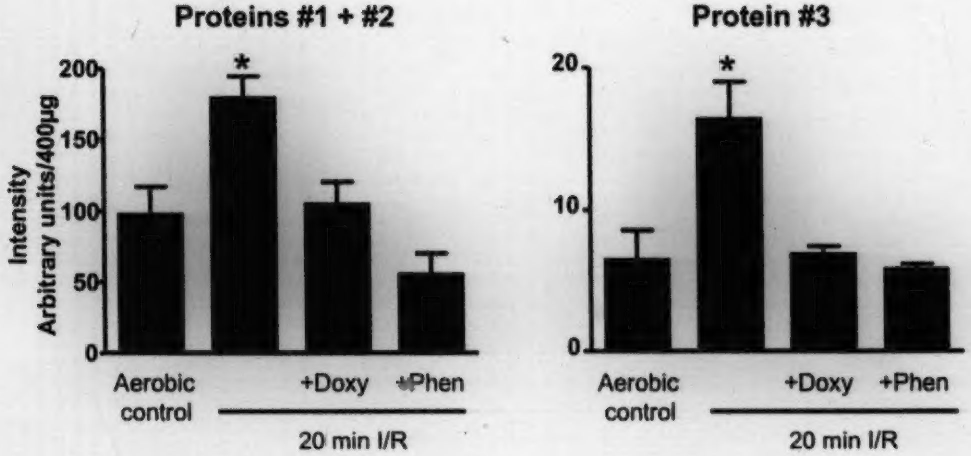

Figure 4.5 Densitometric analysis of identified protein spots. Bar graphs show analysis of the intensities of protein spots 1+2 and protein 3, identified in Figure 4.4 (n=3/group). *p<0.05 vs. Aerobic control, ANOVA.

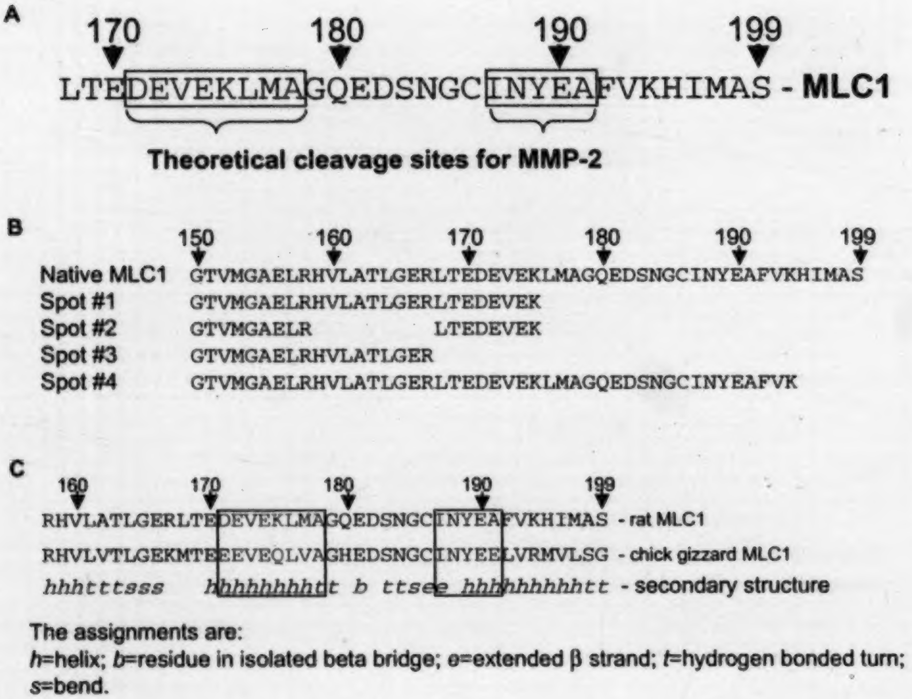
A
170
180
190
199
LTEDEVEKLMAGQEDSNGCINYEAFVKHIMAS - MLC1
Theoretical cleavage sites for MMP-2
B
150
160
170
180
190
199
Native MLC1 GTVMGAELRHVLATLGERLTEDEVEKLMAGQEDSNGCINYEAFVKHIMAS
Spot #1 GTVMGAELRHVLATLGERLTEDEVEK
Spot #2 GTVMGAELR LTEDEVEK
Spot #3 GTVMGAELRHVLATLGER
Spot #4 GTVMGAELRHVLATLGERLTEDEVEKLMAGQEDSNGCINYEAFVK
C
160
170
180
190
199
RHVLATLGERLTEDEVEKLMAGQEDSNGCINYEAFVKHIMAS - rat MLC1
RHVLVTLGEKMTEEEVEQLVAGHEDSNGCINYEELVRMVLSG - chick gizzard MLC1
hhhtttsss hhhhhhhhtt b ttsee hhhhhhhhhtt - secondary structure
The assignments are:
h=helix; b=residue in isolated beta bridge; e=extended β strand; t=hydrogen bonded turn;
s=bend.

Figure 4.6 Theoretical analysis, peptide comparison and structural analysis of cleavage sites of MLC1 by MMP-2. (A) Theoretical analysis for possible MMP-2 cleavage sites within myosin light chain 1 (MLC1). The amino acid sequence of MLC1 was compared to various MMP-2 cleavage recognition sites.[1,2] The boxes show the location of the theoretical cleavage sites with the amino acid sequence represented in different color (magenta and red). (B) Comparison of peptides obtained by MS/MS analysis from all 4 protein spots with intact MLC1 from glycine 151 and onwards in the c-terminal portion. (C) Analysis of the secondary structure of rat MLC1 for structural susceptibility to proteolytical cleavage by MMP-2. The boxes show the location of the predicted cleavage sites and corresponding secondary structure. *(Analysis performed with help from G. Sawicki).*

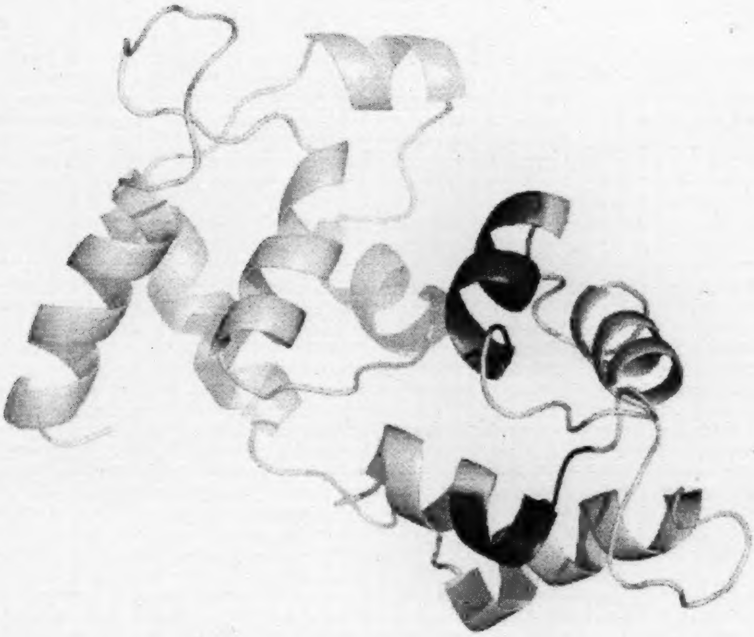

Figure 4.7 Spatial location of predicted cleavage sites in a 3-dimensional model of rat ventricular MLC1. Sequences within which MMP-2 is predicted to cleave (see text) are coloured magenta (residues 171 to 178) and red (residues 187 to 191). *(Computer modeling done with help from PG. Scott).*

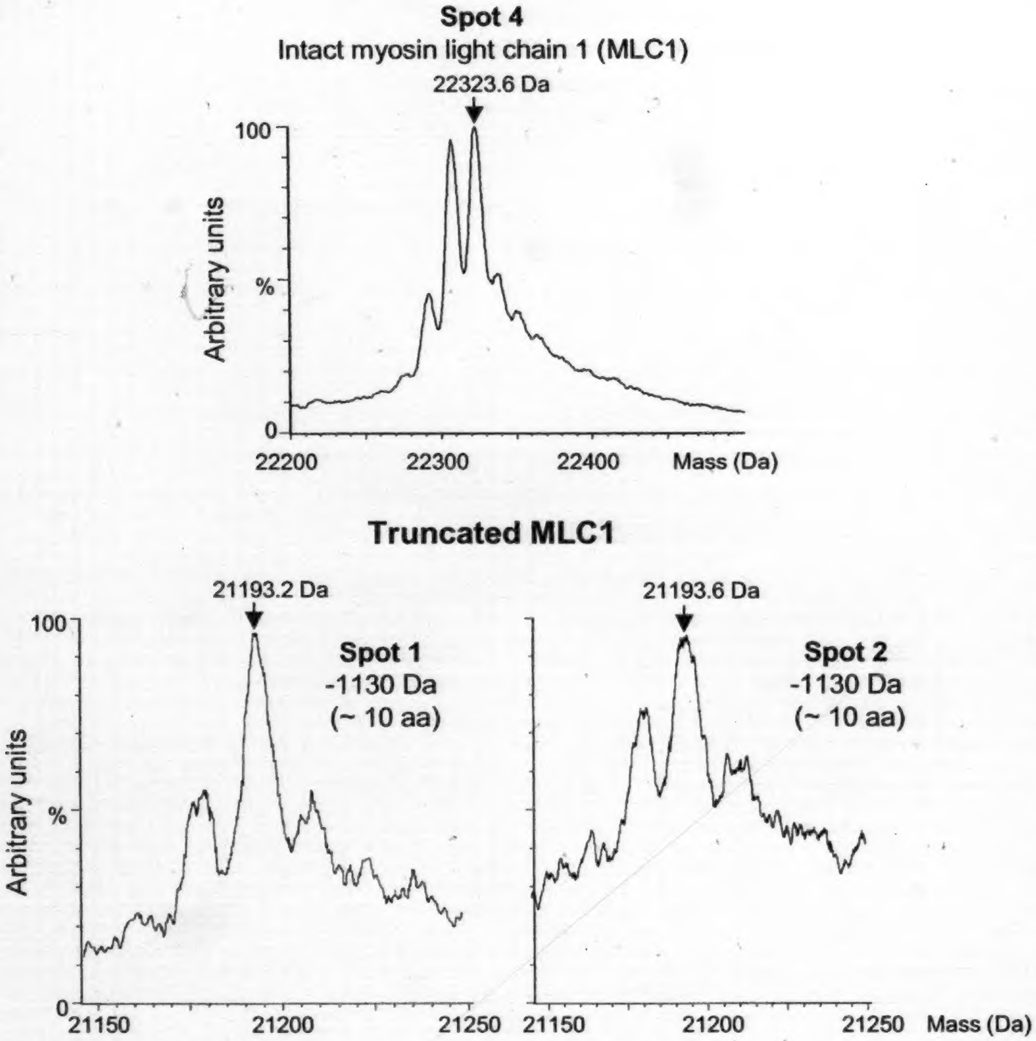

Figure 4.8 Measurement of molecular masses by mass spectrometry of the intact protein spots 1, 2 and 4. Upper panel shows the intact MLC1 protein (Spot 4). Lower panel shows the truncated forms of MLC1, spots 1 and 2 respectively. The arrows show the selected peaks taken for analysis.

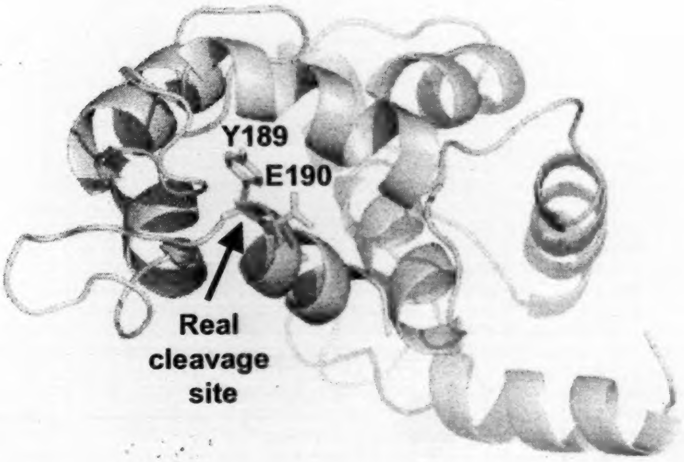

Figure 4.9 Spatial location of detected cleavage site in 3-dimensional model of rat ventricular MLC1. The arrow shows the real cleavage site which is flanked by tyrosine residue (Y189) and glutamic acid residue (E190), shown as sticks. *(Computer modeling done with help from PG. Scott).*

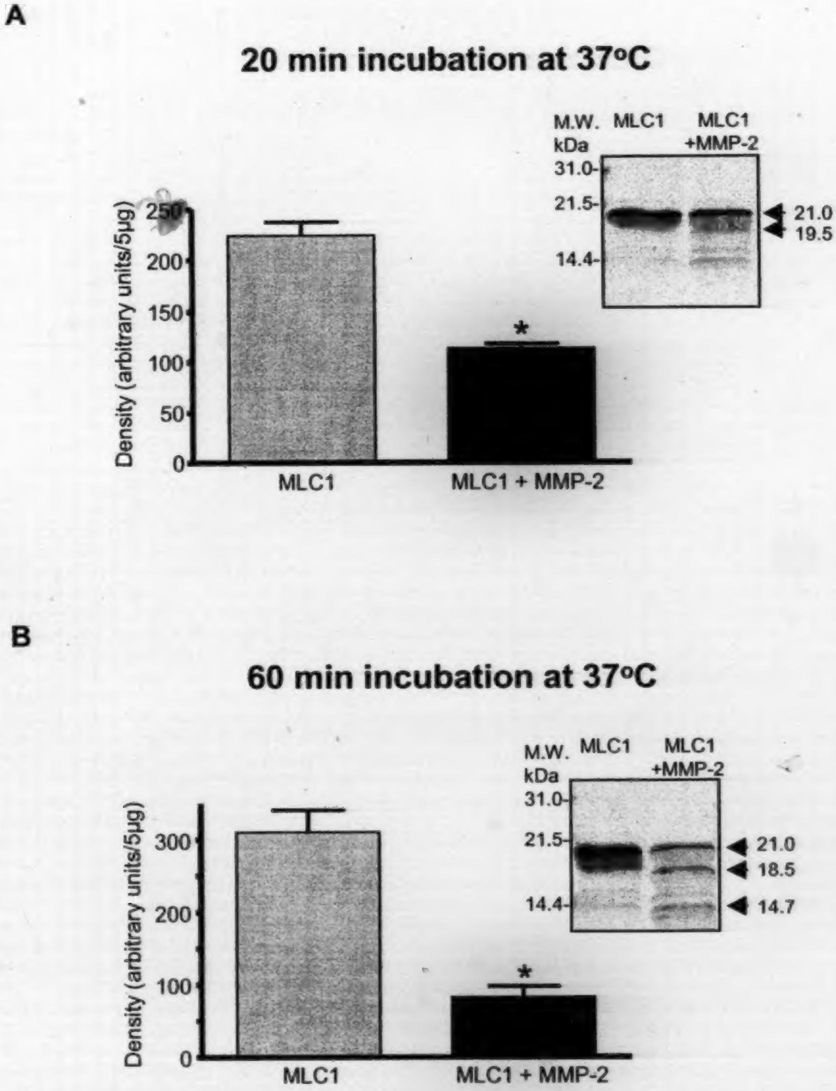

Figure 4.10 ***In vitro*** **degradation of MLC1 by MMP-2 after 20 (A) or 60 min incubation (B).** Staining shows a loss of the 21 kDa MLC1 band with increasing time of incubation and appearance of 19.5, 18.5 and 14.7 kDa degradation products. Bar graphs represent the quantitative analysis of the degradation of MLC1 by MMP-2. $^{*}p<0.05$ vs. MLC1, n = 3. *(Experiments done with help from J. Sawicka).*

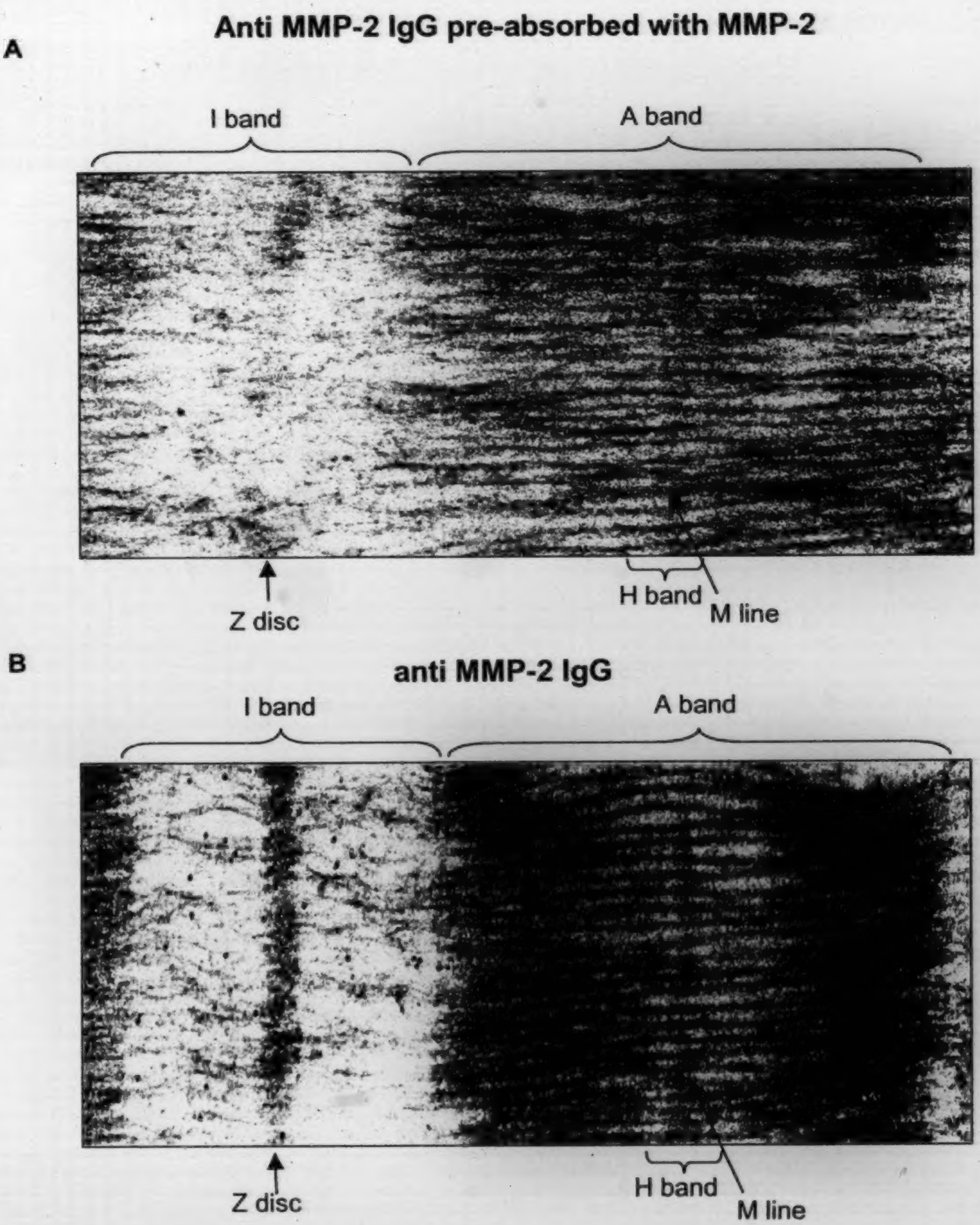
A
Anti MMP-2 IgG pre-absorbed with MMP-2
I band
A band
Z disc
H band
M line
B
anti MMP-2 IgG
I band
A band
Z disc
H band
M line

Figure 4.11 Localization of MMP-2 within the sarcomere of I/R rat hearts by immunogold electron microscopy. Upper panel shows representative microphotograph of negative control with anti MMP-2 IgG pre-absorbed with MMP-2. Bottom panel shows the localization of MMP-2 along the sarcomere, positive staining is shown as the black immunogold dots. *(Experiments done with help from CJ. Schulze).*

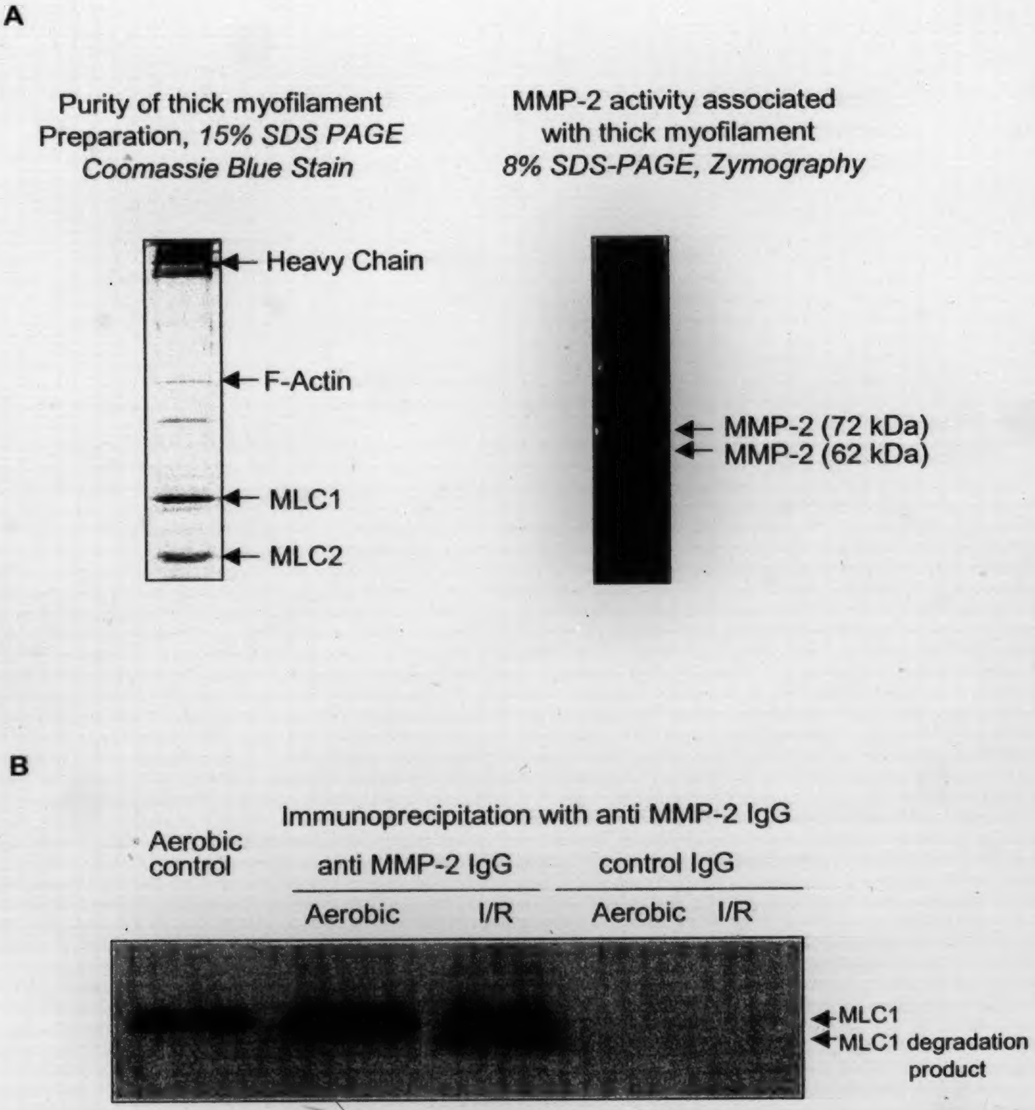
A
Purity of thick myofilament
Preparation, *15% SDS PAGE*
Coomassie Blue Stain
Heavy Chain
F-Actin
MLC1
MLC2
MMP-2 activity associated
with thick myofilament
8% SDS-PAGE, Zymography
MMP-2 (72 kDa)
MMP-2 (62 kDa)
B
Immunoprecipitation with anti MMP-2 IgG
Aerobic
control
anti MMP-2 IgG
control IgG
Aerobic
I/R
Aerobic
I/R
MLC1
MLC1 degradation
product

Figure 4.12 Association of MMP-2 with the thick myofilament. (A) Left panel shows the quality of the thick filament preparation following 15% SDS-electrophoresis. Right panel reveals 72 kDa and 62 kDa gelatinolytic activity (arrows) associated with thick myofilament. (B) Association of MMP-2 with MLC1 in heart homogenates immunoprecipitated with anti MMP-2 IgG. Western blot analysis with anti MLC1 IgG shows that MLC1 associates with MMP-2 in both Aerobic control and I/R heart homogenates. Left lane is homogenate from Aerobic control heart.

Table 4.1 Results of the identification of protein spots 1-4 using Mascot search engine

Protein spot (#)	Probability based on Mowse score*		Peptide matched (n)	Protein identity
	Threshold ($p < 0.05$)	Observed score		
1	41	579	15	MLC1
2	26	477	12	MLC1
3	26	345	8	MLC1
4	26	621	58	MLC1

*-10log(P) where P is the probability that the observed match is a random event. Individual ion scores >26 or >41 indicate identity or extensive homology ($p < 0.05$).

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CHAPTER 5

INHIBITION OF MATRIX METALLOPROTEINASES PREVENTS PEROXYNITRITE-INDUCED CONTRACTILE DYSFUNCTION IN THE ISOLATED CARDIAC MYOCYTE

This work was submitted: León H, Baczko I, Light PE, Sawicki G, Schulz R. Inhibition of matrix-metalloproteinases prevents peroxynitrite-induced contractile dysfunction in single cardiac myocytes. *Journal of Molecular Cell Cardiology.*

5.1 INTRODUCTION

The overproduction of ROS including the potent cellular oxidant $ONOO^-$ can outstrip cellular antioxidant defenses leading to oxidative stress.[1] This condition has been implicated in the pathogenesis of many cardiovascular diseases such as heart failure[2] and ischemia-reperfusion injury.[3-5]

Overproduction of these oxidant species results in the activation of MMPs, enzymes best known for their action in remodeling the extracellular matrix.[6-8] However, recent studies have unravelled new roles of MMPs, particularly MMP-2 which is ubiquitous to most cell types including cardiac myocytes,[9,10] in both physiological and pathological processes such as platelet aggregation, inflammation, and neurodegenerative disease.[11-13] In each of these conditions the biological effect of MMP-2 occurs by its proteolytic action on novel substrates unrelated to extracellular matrix proteins. MMPs are implicated in the pathogenesis of many cardiovascular diseases in both animal models as well as in humans including myocardial infarction,[14-16] heart failure,[17-19] ischemia-reperfusion injury,[10,20-23] cytokine-induced cardiac dysfunction,[24] and sepsis.[25] However, whether an intracellular site of MMP action in mediated contractile dysfunction in cardiac tissue has not been yet determined.

$ONOO^-$ is a potent oxidant which activates MMPs by S-glutathiolation of a critical cysteine residue in their propeptide domain resulting in a full length active MMP.[8] It was previously shown that infusion of $ONOO^-$ into the isolated rat heart causes cardiac contractile dysfunction through MMP-2 which is prevented by MMP inhibition.[26] However, this study did not directly address whether the detrimental effect of MMP-2 occurred via its proteolytic actions on extracellular matrix proteins or on targets specifically associated with the cardiac myocyte.

Ishida *et al*,[27] showed that the direct administration of $ONOO^-$ to isolated neonatal cardiac myocytes caused severe contractile dysfunction, evaluated as the time taken for contractions to cease (contraction cease time, CCT). Whether MMPs are involved in $ONOO^-$ -induced contractile failure in isolated adult cardiac myocytes is unknown. We hypothesized that $ONOO^-$ produces contractile dysfunction through activation of MMPs independent of their action on extracellular matrix proteins.

5.2 METHODS

All the experiments were performed according to the recommendations given by the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care (revised 1993).

5.2.1 Isolation of cardiac myocytes

Calcium tolerant ventricular myocytes were obtained by enzymatic dissociation as previously described.[28,29] Adult male Sprague-Dawley rats (250-350 g) were anesthetized with an injection of sodium pentobarbital (60 mg/kg i.p.). The hearts were isolated and perfused through the aorta in a modified Langendorff perfusion system at constant flow and at 37°C, with oxygenated (95% O_2: 5% CO_2) Krebs-Henseleit solution containing (in mM): NaCl (121), KCl (5), CH_3COONa (1.7), $MgCl_2$ (0.1), Na_2HPO_4 (0.4), $NaHCO_3$ (20.8), glucose (5.5), taurine (1), and $CaCl_2$ (1) for 5 min to clear any blood from the coronary circulation. The solution was then switched to a Ca^{+2}-free Krebs-Henseleit solution for 9 min. After this the solution was replaced for the first digestion process with Krebs-Henseleit solution containing 40 μM Ca^{2+} and 13.3 μg/mL streptomyces collagenase (Yalkut Pharmaceutical, Tokyo, Japan) and 13.3 μg/mL streptomyces protease (Sigma), for approximately 10 min. The ventricles were then separated from the atria and great vessels using scissors and chopped into small pieces. For the second digestion process the chopped tissue was placed into a flask with Krebs-Henseleit solution containing 5.5 mM HEPES, 3% bovine serum albumin, 100 μM Ca^{2+} and 83.3 μg/mL collagenase and 83.3 μg/mL protease. The cells were further dissociated by incubation at 37°C under gentle agitation. A 2 mL aliquot of cell suspension was removed after 10, 20, 30 and 40 min incubation and centrifuged for 25 seconds at 2000 g. 2 mL of the second digestion buffer was replaced in the flask each time after sampling. Each cell suspension was immediately centrifuged and the cells were resuspended in storage buffer (Krebs-Henseleit solution containing 5.5 mM HEPES, 3% bovine serum albumin and 100 μM Ca^{2+}). The aliquots were observed under a light microscope for morphology and aliquots which showed more than 70% rod shaped cells (typically after 30 min digestion) were used for experiments.

5.2.2 Synthesis of peroxynitrite ($ONOO^-$)

Active and decomposed $ONOO^-$ were synthesized and the concentration was verified on the same day of experiments using UV spectroscopy as described previously.[3] Both were diluted with 1 mM NaOH immediately prior to the experiment in order to achieve the desired working concentration.

5.2.3 Measurement of myocyte contractility

In order to maintain a constant $ONOO^-$ to myocyte ratio, approximately 5000 cardiac myocytes were placed into a perfusion chamber in each experiment. Experiments were performed at 21°C. Freshly isolated rat ventricular myocytes were continuously superfused at 1 mL/min with Krebs-Henseleit solution with 2 mM Ca^{2+} using an infusion pump (Cole-Palmer, Barrington, IL) and electrically paced at 0.5 Hz. Single myocyte contractility was measured using a video edge-detector (Crescent Electronics, Salt Lake City, UT) and data recorded using pClamp 8.0 software. After three minutes superfusion with Krebs-Henseleit for recording baseline parameters, an infusion over 40 min (or until the cells ceased their contractions) of either decomposed or active $ONOO^-$ was started via a side-arm using a microinfusion pump (Baxter, Deerfield, IL) to reach a final concentration of 30, 100, or 300 μM in order to evaluate the contraction cease time.

Another series of isolated cardiac myocytes were subjected to two different MMP inhibitors in the absence of $ONOO^-$ in order to evaluate possible effects of these compounds on contractility. After the 3 min baseline recording, the superfusion buffer was replaced to the same Krebs-Henseleit buffer containing either doxycycline (100 μM, Sigma) or PD 166793 (2 μM, a kind gift from Pfizer) and the contractile function was monitored for 10 min. Contractility parameters measured included cell shortening (% of cell shortening vs. original cell size during contraction), and the time to 50% relaxation (time required for the cell to achieve 50% of relaxation after systole), the latter as a measure of diastolic function. Parallel experiments were performed using their respective vehicles (water for doxycycline and 0.05% v:v DMSO for PD166793). These MMPs inhibitors and the concentrations employed were chosen based on previous data from isolated rat hearts subjected to either I/R,[10,20-22] pro-inflammatory cytokines[24] or exogenous ONOO$^-$.[26]

A further series of experiments was performed in order to evaluate the effect of MMP inhibitors on CCT during ONOO$^-$ challenge. After the 3 min baseline recording, the perfusion buffer was changed to the one containing either doxycycline or PD 166793 or their respective vehicles.

5.2.4 Evaluation of MMP activity

The cell storage buffer was removed from an aliquot of freshly isolated cardiac myocytes and replaced with Krebs-Henseleit buffer containing 100 μM Ca^{2+} (oxygenated with 95% O_2: 5% CO_2) to reduce hypercontracture by three separate wash and centrifugation steps (2000g for 25 s). Cardiac myocytes (~ 200,000 cells) were then exposed to the bolus addition of either 300 μM ONOO$^-$ or decomposed ONOO$^-$ followed by 5 min incubation at 21°C. Cells were separated from the media by centrifugation (2000g for 40 s) and the latter was concentrated 20 times using Amicon Ultra-4 centricon tubes (Millipore, Bedford, MA). Gelatin zymography was performed as described.[20] Samples from cell homogenates and media were applied to 8% polyacrylamide gels copolymerized with 2 mg/mL gelatin. After electrophoresis, gels were rinsed three times for 20 min each in 2.5% Triton X-100 in order to remove SDS. Then the gels were washed twice in incubation buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 150 mM NaCl and 0.05% NaN_3) for 20 min each at room temperature and then incubated in a fresh aliquot of the same at 37°C. The gels were stained in 2% Coomassie Brilliant Blue G, 25% methanol, 10% acetic acid for 2 hr and then destained for 1 hr in 2% methanol/4% acetic acid. Gels were scanned using a GS-800 calibrated densitometer (Bio-Rad) and band densities were measured using SigmaGel software (Jandel)

5.2.5 Evaluation of intracellular [Ca^{2+}]

Cardiac myocytes placed in storage buffer were loaded for 30 min at room temperature and then 30 min at 37°C with the Ca^{2+}-sensitive fluorescent probe calcium green-1 AM (4 μM, dissolved in a mixture of dimethyl sulfoxide and pluronic acid, 1:1 v/v, Molecular Probes, Eugene, OR). After loading, cells were washed and centrifuged twice (2000g for 25 s) with storage buffer and placed on coverslips for observation at 200X with an inverted microscope (Olympus, CK40) while being superfused with the same Krebs-Henseleit buffer used for contractility measurements, and paced at 0.5 Hz at 21°C. An infusion of either decomposed or 300 μM $ONOO^-$ was started after the baseline recording over a period of 20 min via a side-arm using a microinfusion pump. Some cardiac myocytes were exposed to doxycycline (100 μM) dissolved in Krebs-Henseleit buffer 5 min before starting the infusion of 300 μM $ONOO^-$ and maintained during the 20 min exposure period. A Photon Technology International (Model 814, Lawrenceville, NJ) photomultiplier detection system and Clampex software (version 8.1) was used for data acquisition and analysis. Calcium Green-1 AM was excited at 480 nm and the emitted light intensity at 520 nm was digitized and stored. The parameters evaluated were the time to the maximal increase in diastolic and systolic calcium, and the percentage increase in maximum diastolic calcium.

5.2.6 Statistical Analysis

Data are expressed as means ± SE. Student's t-test (unpaired) or one-way ANOVA followed by post-hoc analysis using Tukey's multiple comparison test were used for statistical analysis, as appropriate. $p < 0.05$ was the criterion for significance. The n number refers to the number of individual cardiac myocytes tested. In all experiments cells were tested from ≥4 separate isolations of myocytes.

5.3 RESULTS

5.3.1 Concentration-dependent contractile dysfunction by ONOO$^-$

In order to evaluate contractile dysfunction in the isolated cardiac myocyte, we used the contraction cease time (CCT) as a parameter of mechanical function. This parameter was previously used in neonatal cardiac myocytes exposed to ONOO$^-$.[27] There was a progressive decrease of CCT with increasing concentrations of ONOO$^-$ (Figure 5.1). The administration of decomposed ONOO$^-$ did not affect the CCT vs. control (32.5 ± 3.5 vs. 35.1 ± 3.0 min; $p > 0.05$, $n = 8$ and $n = 9$ respectively). There was no change in the pH of the perfusion solution at concentrations of ONOO$^-$ up to 300 μM, or with decomposed ONOO$^-$.

5.3.2 MMP inhibitors do not alter baseline contractile properties of isolated cardiac myocytes

Cardiac myocytes were treated with two different MMPs inhibitors (doxycycline or PD 116793) as well as their respective vehicle controls (water for doxycycline and 0.05% DMSO for PD 166793; n=4). During the 10 min perfusion period cells contracted regularly and none of the cells ceased to contract. Moreover, there was no evidence of early or after depolarizations in the traces nor any significant differences in cell shortening or the time to 50% relaxation (data not shown).

5.3.3 MMP inhibitors prevent contractile dysfunction caused by ONOO$^-$

Cardiac myocytes were exposed to a continuous infusion 300 μM ONOO$^-$ in the presence or absence of the MMPs inhibitors (doxycycline or PD 166793), or their respective vehicles. Administration of 300 μM ONOO$^-$ caused a rapid onset in contractile dysfunction in myocytes which was revealed as irregular contractions with early after-depolarizations and hypercontracture as the infusion of ONOO$^-$ progressed (Figure 5.2A). Myocytes challenged to 300 μM ONOO$^-$ in the presence of doxycycline or PD 166793 exhibited a delayed onset of contractile dysfunction in comparison to ONOO$^-$ alone (Figure 5.2B and 2C). Summary data shows that doxycycline or PD 166793 significantly prolonged the CCT (Figure 5.3).

5.3.4 Impairment in Ca^{2+} homeostasis caused by ONOO$^-$ is unaffected by MMPs inhibitors

In order to evaluate whether the protective effect of MMPs inhibition was related to possible effects on Ca^{2+} homeostasis we measured this in cardiac myocytes subjected to 300 μM ONOO$^-$. Ca^{2+} homeostasis in cardiac myocytes exposed to decomposed ONOO$^-$ was stable over the duration of the experimental protocol (Figure 5.4A). In contrast, 300 μM ONOO$^-$ caused a progressive increase in diastolic [Ca^{2+}] as well as reduced peak systolic transient amplitude (Figure 5.4B). Doxycycline treatment delayed the onset of changes in Ca^{2+} homeostasis but did not prevent them (Figure 5.4C).

Represented in terms of the time to reach maximum diastolic [Ca^{2+}] during the experimental protocol, cardiac myocytes challenged with 300 μM $ONOO^-$ showed a reduced time to maximum increase in diastolic [Ca^{2+}] level compared with decomposed $ONOO^-$. Doxycycline, however, did not alter this (Figure 5.5A). A similar finding was observed for the time to reach the peak in systolic [Ca^{2+}] level (Figure 5.5B). The percent increase in maximal diastolic [Ca^{2+}] (Figure 5.5C) tended to be higher in $ONOO^-$ exposed cells vs. decomposed $ONOO^-$, however, this difference did not reach the statistical difference (65 ± 29% vs.-2 ± 10%; p= 0.06, n=4). Doxycycline had no further effect on this parameter (Figure 5.5C).

5.3.5 $ONOO^-$ activates myocyte MMP-2

We tested whether $ONOO^-$ was able to activate MMPs when given as a bolus to an aliquot of cardiac myocytes. Gelatin zymography was performed on the concentrated incubation media from cardiac myocytes in order to examine changes in MMP-2 or MMP-9 activities. After 5 min exposure to 300 μM $ONOO^-$, there was a significant increase in 62 kDa MMP-2 activity in the media in comparison to decomposed $ONOO^-$ and no evidence of 72 kDa activity (Figure 5.6). We did not observe any evidence of MMP-9 activity (data not shown).

5.4 DISCUSSION

This study is the first demonstration that inhibition of MMPs reduces $ONOO^-$-induced contractile dysfunction in cardiac myocytes independent from the actions of MMPs on extracellular matrix proteins. Previously we demonstrated that MMP-2 is activated in isolated rat hearts challenged with $ONOO^-$ and that a MMP inhibitor prevented this dysfunction.[26] However, this study did not determine whether the effect of the activation of MMP-2 was independent of proteolysis of extracellular matrix proteins.

During myocardial ischemia-reperfusion injury $ONOO^-$ is rapidly generated during the first minutes of reperfusion which precedes the activation and release of MMP-2 from the heart.[3,20] In the current study we observed an increase in 62 kDa MMP-2 activity in the conditioned cell medium following exposure to $ONOO^-$. The release of MMP-2 from heart muscle is understood to be a consequence of its activation by oxidative stress[10,20,26] and may be a means for the cell to protect itself from further proteolytic stress. MMP-2 activation as a result of ischemia-reperfusion injury in the intact heart results in its cleavage of troponin I and myosin light chain 1.[10,22] Whether the same event occurs during $ONOO^-$ exposure to the isolated myocytes is unknown. Our data clearly show that the effect of $ONOO^-$ was mediated by MMP activity as the contractile dysfunction was diminished by two structurally independent MMPs inhibitors, doxycycline or PD 166793, and was independent of an action of MMPs to cleave extracellular matrix proteins in the isolated myocytes studied here.

$ONOO^-$ can activate MMPs by different mechanisms. One of these is the S-glutathiolation of the pro-peptide domain which was observed for MMP-1, -8 and –9.[8] Moreover, it was suggested that activation of MMPs by $ONOO^-$ could be mediated by nitration of tyrosine residues in the hinge region and further unfolding of the pro-MMP[6] or by S-nitrosylation of the cysteine residue in the pro-peptide domain as occurs with MMP-9 when is activated by either ischemia-reperfusion or following nitric oxide donor administration.[31] The effect of $ONOO^-$ on MMP activity is biphasic as higher concentrations of $ONOO^-$ have clearly been shown to inactivate MMPs.[8,32] On the other hand, $ONOO^-$ was shown to inhibit tissue inhibitors of MMPs (TIMP-1 and TIMP-2) which could increase net gelatinolytic activity in the cells.[33,34] Due to the lack of a technique which could evaluate the activation of MMPs inside the cell in real time we could not observe the intracellular compartment(s) in which MMPs activation occurred upon exposure to $ONOO^-$. Future studies and technical development are required to address this question.

$ONOO^-$ may cause cellular injury through several mechanisms including protein modifications and the inactivation of proteins involved in Ca^{2+} handling, such as the nitration and inactivation of sarcoplasmic reticulum Ca^{2+} ATP-ase in porcine cardiac sarcoplasmic reticulum microsomes with either 300 μM or 1 mM $ONOO^-$.[35] $ONOO^-$ inhibited the Na^+/Ca^{2+} exchanger in microsomes prepared from bovine smooth muscle cells, an event accompanied by the activation of MMP-2 and inhibition of tissue inhibitor of metalloproteinase-2 (TIMP-2).[34] Moreover, reactive oxygen species have deleterious effects on Ca^{2+} homeostasis in cardiac myocytes through their action in a number of proteins including the Na^+/Ca^{2+} exchanger, phospholamban and calcium channels.[36-39]

Ishida *et al*,[27] showed that infusion of 200 μM $ONOO^-$ caused contractile dysfunction in isolated neonatal cardiac myocytes and this effect was accompanied by an impaired Ca^{2+} homeostasis. In our model using freshly isolated adult cardiac myocytes we also observed abnormalities in Ca^{2+} homeostasis due to $ONOO^-$. The MMPs inhibitor doxycycline delayed the onset but did not prevent the impairment in Ca^{2+} homeostasis caused by $ONOO^-$.

In conclusion we demonstrated that inhibition of MMPs prevents contractile dysfunction in isolated cardiac myocytes resulting from their direct exposure to $ONOO^-$. The effect of MMP activation in these cells is independent of a proteolytic action on extracellular matrix proteins. This provides further insight into the pathological mechanisms related to oxidative stress myocardial injury. Detoxifying $ONOO^-$ or inhibiting the activation of MMPs or their enzymatic activity could lessen the impact of oxidative stress in cardiac disease.

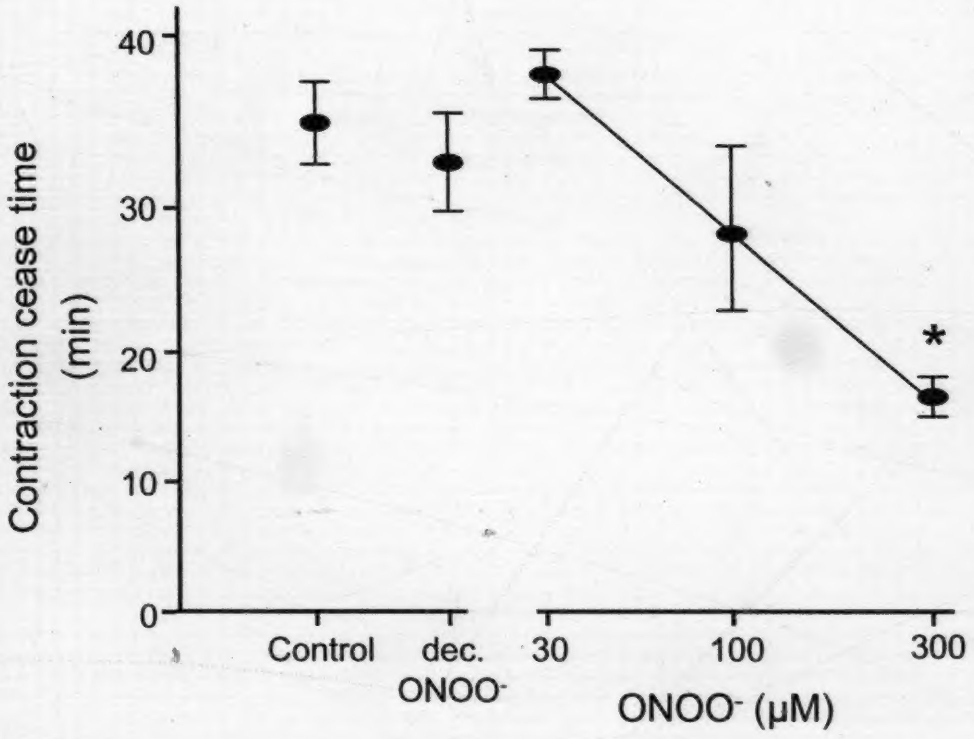

Figure 5.1 Concentration-dependent effect of 40 min continuous infusion of $ONOO^-$ on contraction cease time (CCT) in isolated cardiac myocytes. *$p<0.05$ vs. Control, n =4-9 cells from a total of 6 independent isolations.

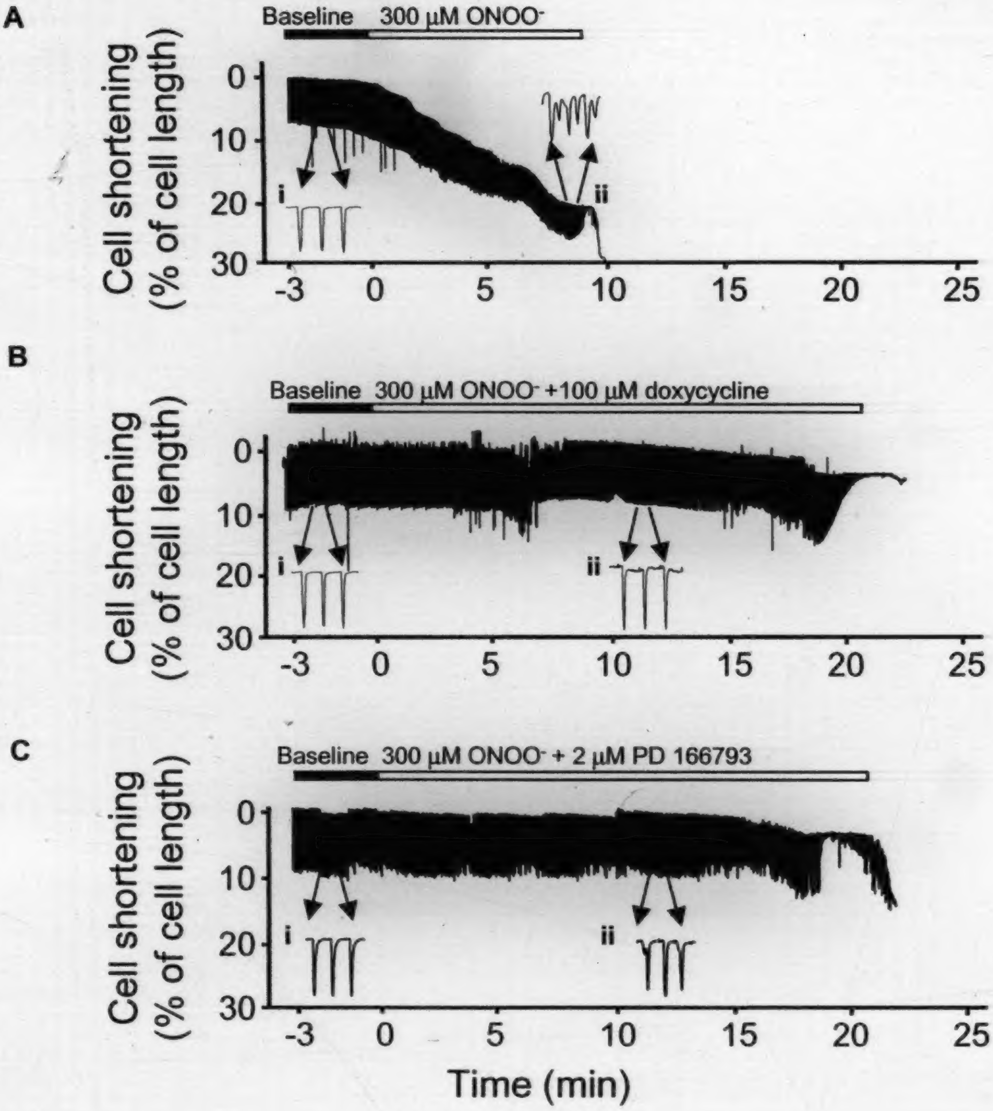

Figure 5.2 Effects of MMPs inhibitors on contraction cease time during 300 μM ONOO⁻ exposure. Panels A-C depict representative traces of cell shortening in cardiac myocytes subjected to ONOO⁻ alone (A) or with 100 μM doxycycline (B), or 2 μM PD 166793 (C). In each panel the expanded time scale of the cell shortening traces are shown at: i) baseline recording, and ii) 10 min after infusion of 300 μM ONOO⁻.

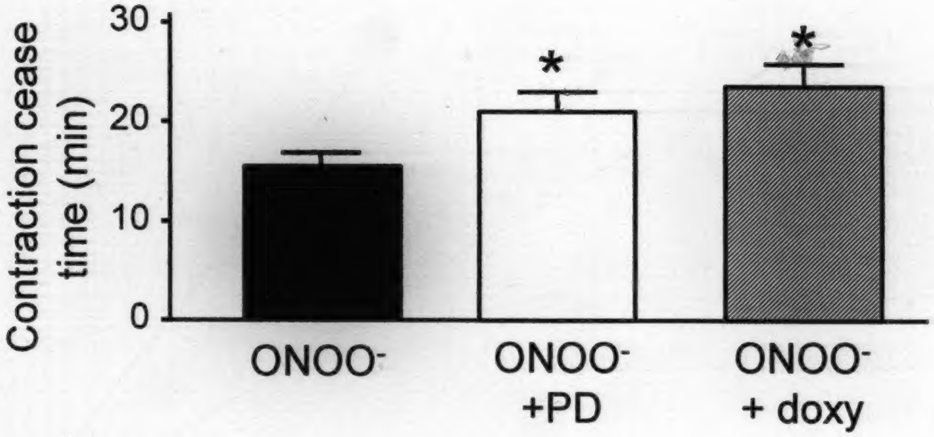

Figure 5.3 Summary analysis in terms of the contraction cease time. n = 5-6 cells/group from a total of 5 independent isolations. *p<0.05 vs. $ONOO^-$.

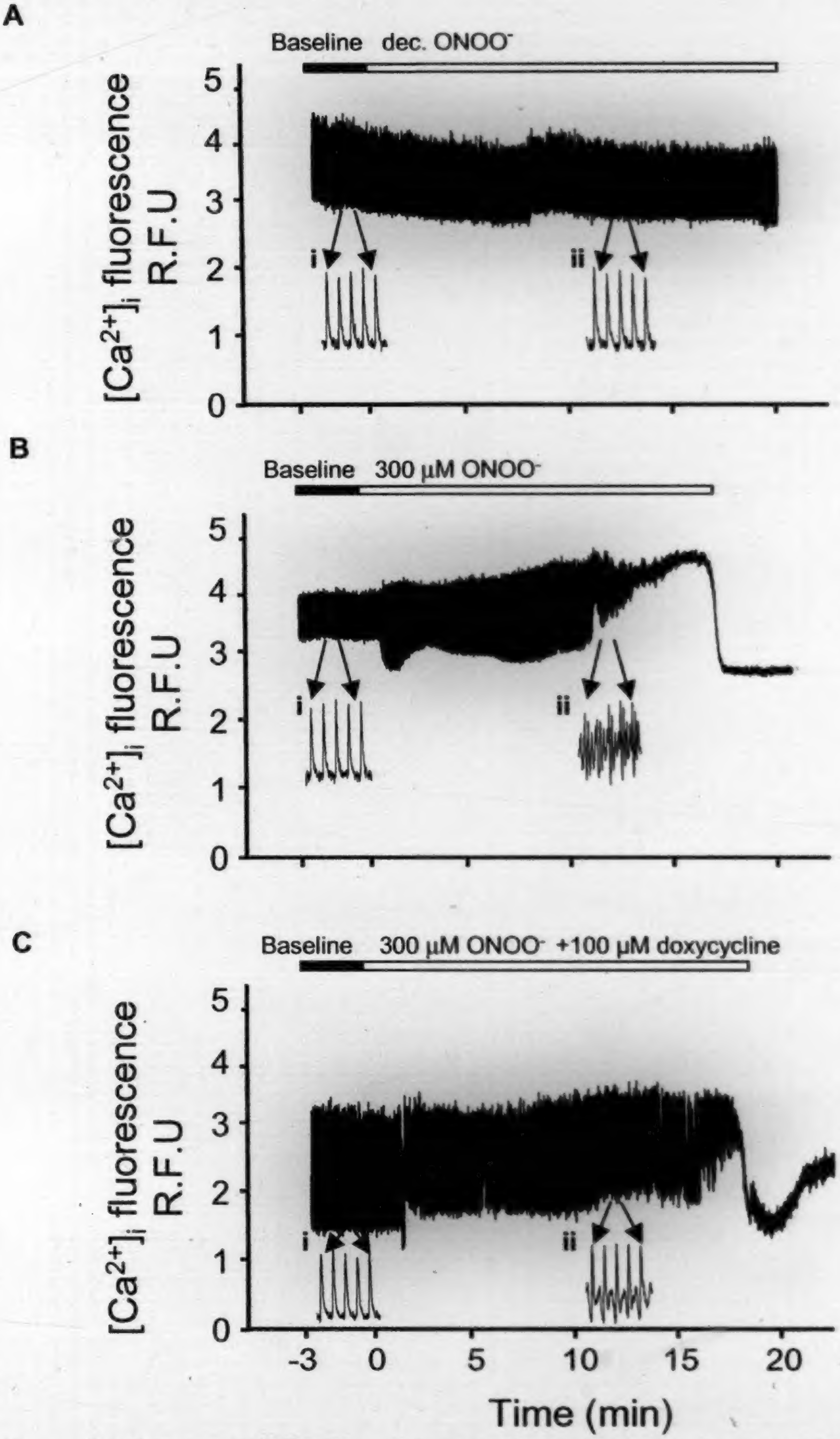

Figure 5.4 Effects of ONOO$^-$ and doxycycline on calcium homeostasis. Representative traces of cells exposed to either decomposed ONOO$^-$ (A) or 300 μM ONOO$^-$ alone (B), or with addition of 100 μM doxycycline (C). Expanded time scale traces of calcium transients at: i) baseline recording, and ii) 10 min after infusion of ONOO$^-$.

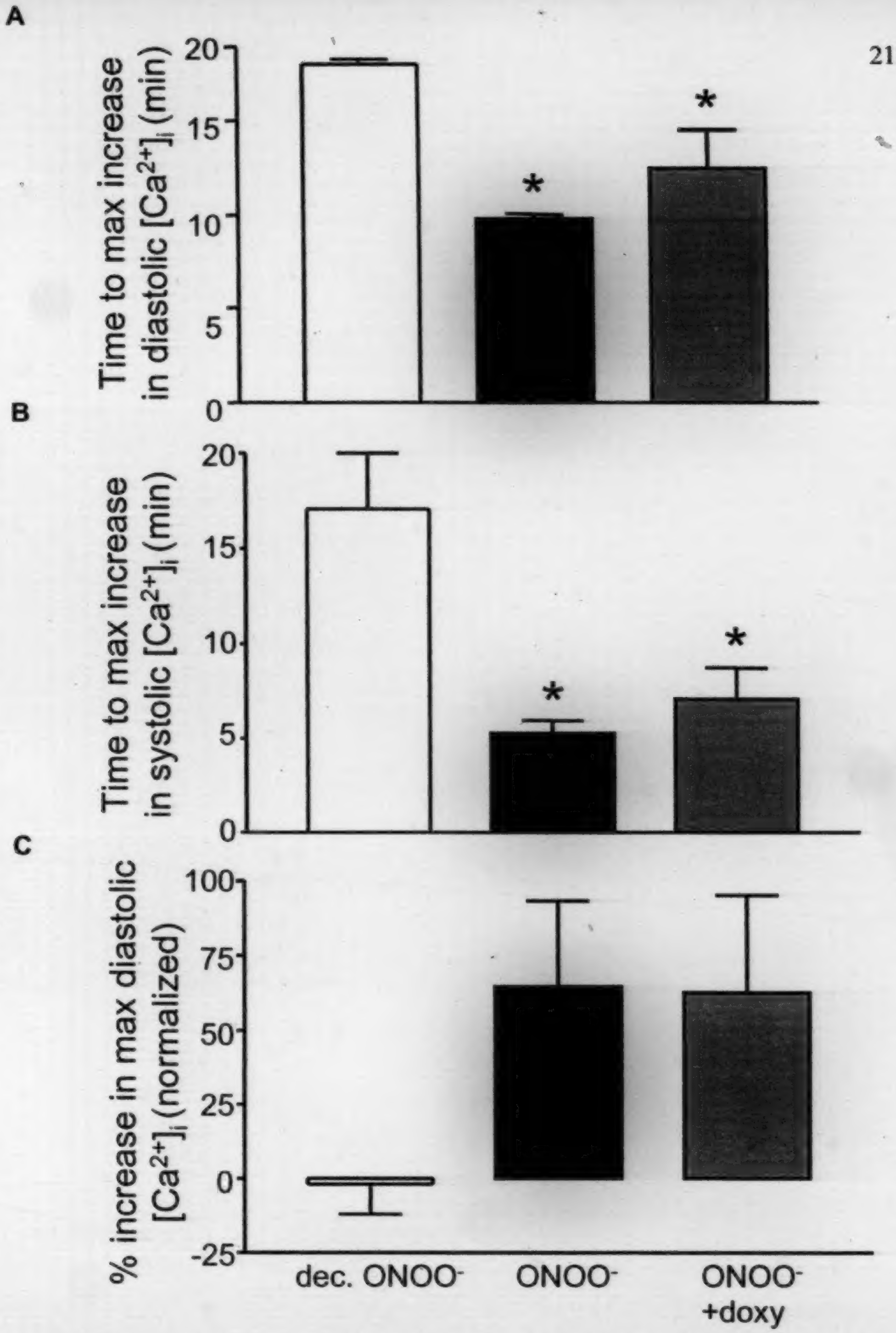

Figure 5.5 Quantitative analysis of the effects of $ONOO^-$ and doxycycline on calcium homeostasis. (A) Time to reach the maximum increase in diastolic calcium level. (B) Time to reach maximal systolic calcium. (C) Percent increase in maximum diastolic calcium level. $^*p < 0.05$ vs. decomposed $ONOO^-$, n=4 cells/group from a total of 4 independent isolations.

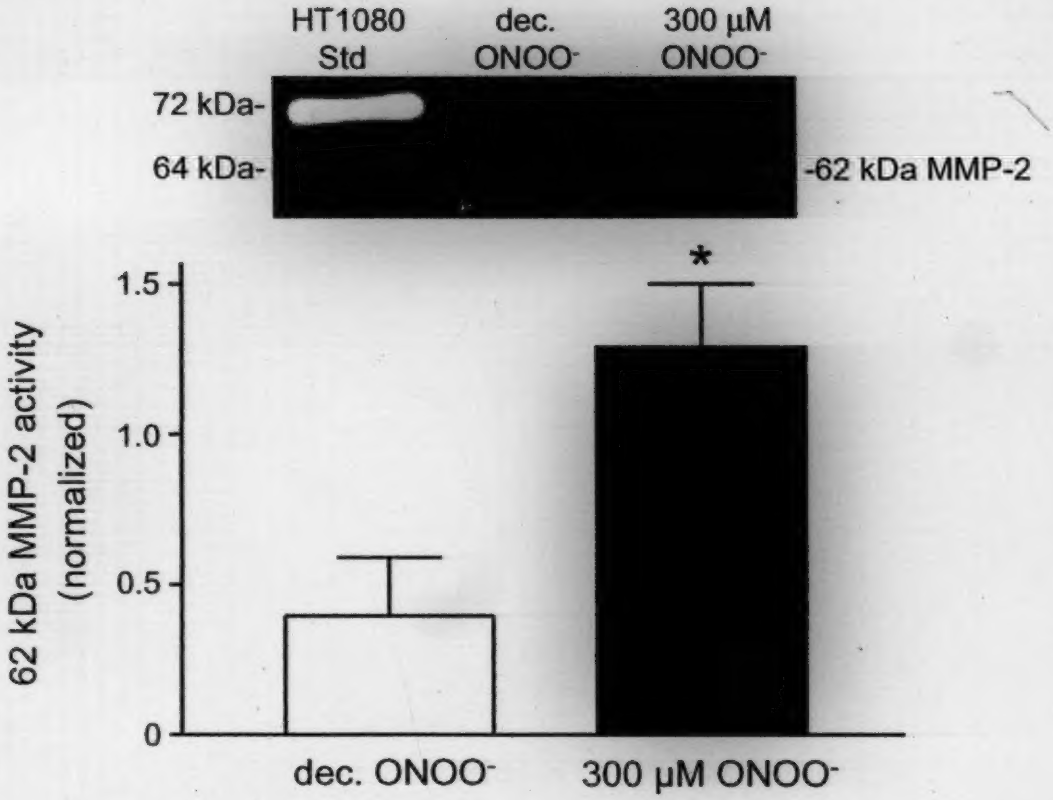

Figure 5.6 Gelatin zymography of media from cardiac myocytes exposed to either decomposed ONOO$^-$ or 300 µM ONOO$^-$. Upper panel shows representative zymogram. Lower panel shows summary data of the densitometric analysis of 62 kDa MMP-2 activity. Note that the HT1080 cell supernatant used as a control is human MMP-2 which is comprised of both 72 kDa and 64 kDa forms, the latter which is slightly higher in molecular weight than the 62 kDa rat MMP-2. *$p < 0.05$ vs. dec. ONOO$^-$, n=3 experiments.

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CHAPTER 6

DOXYCYCLINE PROTECTS ISOLATED CARDIAC MYOCYTES FROM ONOO$^-$-INDUCED OXIDATIVE STRESS

6.1 INTRODUCTION

The enhanced production of ROS is a common feature in the pathogenesis of cardiovascular diseases such as I/R and heart failure.[1-4]

ROS overproduction results in the activation of MMPs, enzymes best known for their action in remodeling the extracellular matrix.[5-7] Recently, it has been reported that MMP-2 in particular has novel roles in physiological and pathological processes. These novel biological roles of MMP-2 are related to its proteolytic action on novel substrates unrelated to extracellular matrix proteins.[8-10] MMP activation is linked to the pathogenesis of many cardiovascular diseases such as myocardial infarction,[11-13] I/R injury,[14-18] heart failure,[19-21] sepsis[22] and cytokine-induced heart dysfunction[23] in both animals and humans. Interestingly, infusion of $ONOO^-$ in isolated rat hearts causes contractile dysfunction by a mechanism involving MMP-2 activation.[24] This event was prevented by scavenging $ONOO^-$ with either glutathione or MMP inhibitors.[24] However, this study did not address whether the detrimental effect of MMP-2 occurred via its proteolytic action on extracellular or intracellular proteins.

Direct administration of $ONOO^-$ to isolated neonatal rat cardiac myocytes led to contractile dysfunction evaluated as the time taken for contractions to cease (contraction cease time, CCT).[25] However, this study did not test whether this phenomenon is related to MMP activation. In Chapter 5, I reported that inhibitors of MMPs attenuated contractile dysfunction (measured as CCT) in adult rat cardiac myocytes. However, whether MMPs played a role in this kind of injury through proteolysis of its known contractile protein targets (troponin I and MLC1) found using intact hearts is unknown. Therefore, we hypothesized that $ONOO^-$-mediated contractile dysfunction in isolated adult rat cardiac myocytes is related to the proteolysis of the contractile proteins troponin I and/or MLC1 by MMPs.

6.2 METHODS

All the experiments were performed according to the recommendations given by the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care (revised 1993).

6.2.1 Synthesis of peroxynitrite ($ONOO^-$)

Active and decomposed $ONOO^-$ were synthesized and the concentration was verified on the same day of experiments using UV spectroscopy as described previously.[2] Both forms of $ONOO^-$ were diluted with 1 mM NaOH immediately prior to the experiment in order to achieve the desired working concentration.

6.2.2 Isolation of cardiac myocytes

Calcium tolerant ventricular myocytes were obtained from adult rats by enzymatic dissociation as previously described in section 5.2.1. Cell viability was immediately assessed by trypan blue exclusion at the end of the cell isolation procedure the value obtained was termed “Baseline”. Cells were kept in storage buffer at 21°C for 1 hr and then the experiment was started.

6.2.3 Experimental protocol-treating cells with $ONOO^-$

The storage buffer was removed from the cells and replaced with Krebs-Henseleit (21°C) buffer containing 100 μM Ca^{2+} (oxygenated with 95% O_2: 5% CO_2) to reduce hypercontracture by three separate wash and centrifugation steps (2000 g for 25 s). Then cells were split into four equal aliquots (~350,000 cells/group) placed in 5 mL sterile cell culture tubes, a fresh aliquot of Krebs-Henseleit buffer used for washing was added to achieve a final volume of 4.2 mL. The cells were kept at 21°C with continuous bubbling with 95% O_2: 5% CO_2. One group of cells was incubated for 10 min with 100 μM doxycycline. After this 10 min incubation period, two groups of cells (including the one incubated with doxycycline) were challenged with 300 μM $ONOO^-$ administered as a single bolus (37.1 μL of a 33,996 μM $ONOO^-$ stock solution). Immediately upon adding the bolus, cells were mixed by hand using gentle agitation, and were incubated at room temperature for five minutes (with bubbling). One group was treated in the same manner with decomposed $ONOO^-$ (dec. $ONOO^-$) and the remaining group was kept as a time control. After the interventions an aliquot (~100,000 cells) from each sample was taken and cell viability was assessed by trypan blue exclusion. The remaining ~250,000 cells/sample were immediately placed on ice and then used for cell media and cell lysate preparation.

6.2.4 Cell media and cell lysate preparation

The remaining cell suspension was centrifuged (2000 g for 40 s) in order to separate the media from the pellet. The media was removed and concentrated 20 times using Amicon Ultra-4 centricon tubes (Millipore) at 4°C and then stored in –80°C. 350 μL of homogenization buffer containing 50 mM Tris-HCl (pH 7.4) containing 3.1 mM sucrose, 1 mM DTT (Fisher Scientific), 1:1000 protease cocktail inhibitor (Sigma P-8340), 0.1% Triton X-100, and 5 mM EDTA was added to the pellet and it was then homogenized on ice using a sonicator (Heat-Systems-Ultrasonics, 3 x 10 s with 60 s between each cycle). The cell lysates were stored at –80°C until used for biochemical assays.

6.2.5 Evaluation of MMP activity

Protein concentration was determined by the Bradford protein assay.[26] Cell media (8 μg/protein) or cell lysate (5 μg/protein) were subjected to gelatin zymography as described.[14] See section 5.2.4.

6.2.6 Evaluation of proteolytic activity of cell lysates using OmniMMP substrate

OmniMMP (Biomol) is synthetic peptide containing the sequence Mca-proline-leucine-glycine-leucine-Dpa-alanine-arginine-NH_2.AcOH (Mca: [methoxycoumarin-4-yl]acetyl; DPA: N-3-[2,4-dinitrophenyl-L-α,β-diaminopropionyl) which can be used to detect MMP activitiy. When it is proteolyzed by MMP activity internal quenching of the fluorophore is diminished and a fluorescent signal is obtained. In order to establish a protein and substrate concentration to evaluate MMPs activity in cell lysates, the hydrolysis of OmniMMP fluorogenic substrate (0-25 μM, prepared in 1.8% (v/v) DMSO) by cell lysates (1.875, 3.75, and 7.5 μg cell lysate protein/reaction) was measured at 37°C in a continuous plate reader-based protocol. Assays were performed in triplicate in a total volume of 120 µL in black polystyrene half area plates (Corning), and contained cell lysate (brought to 60 µl total volume with 2x reaction buffer: 150 mM NaCl, 10 mM HEPES pH 7.5, 5 mM $CaCl_2$, 0.1% Triton X-100, 5 µg/mL sodium azide) and substrate (60 µL at 2x final concentration, prepared in 0.5% v/v DMSO) or DMSO (0.5%, v/v; 60 µl in blank wells). In experiments where MMP inhibitor was included, only 50 µL of substrate (OmniMMP) was added and the inhibitor was included in a volume of 10 µL. Fluorescence associated with a (7-methoxycoumarin-4-yl)acetyl-tagged cleavage product was measured every 30 seconds for 1 hour (λ_{ex} 328 nm, λ_{em} 393 nm) in a Molecular Devices SPECTRAmax Gemini XPS fluorescence microplate reader. The rate of product formation in each well was determined through linear regression of the fluorescence-time data by the plate reader software (SOFTmax Pro, v 4.8; Molecular Devices Inc.). Appropriate lag times, to preclude data obtained prior to equilibration at 37°C, and end times, to preclude data obtained following a loss of linearity, were entered manually prior to linear regression of data to obtain slopes. Rate values were then corrected for loss of signal due to absorption by substrate at 393 nm using a measured substrate extinction coefficient of 7627 $M^{-1}cm^{-1}$ and a measured path length of 0.672 cm (See Figure 6.1).

Based on the results of Figure 6.1 which concludes that the most optimal concentration of substrate and amount of cell lysate to perform this assay are 15 µM and ~3 µg respectively. In order to determine whether the metabolism of the OmniMMP fluorogenic substrate was mediated either totally or partially through MMP activity, the hydrolysis of the substrate (15 µM) by cell lysates (3 µg in 60 µL) was evaluated in the presence of a range of concentrations of the MMPs inhibitor GM 6001 (0.1 nM-100 µM, Calbiochem) at 1/3 log unit intervals of concentration. Neither GM6001 nor DMSO vehicle interferes with the fluorescence at the indicated wavelengths over the concentration range studied in assays performed using recombinant MMP-2 (data not shown).

The metabolism of 15 μM OmniMMP by cell lysates (3 μg in 60 μL) from samples treated with either dec. $ONOO^-$ or active $ONOO^-$ in the presence or absence of doxycycline (100 μM), as well as the Control group was also determined under the conditions described above. The percentage MMP-related activity was determined by subtracting the fluorescence value in a duplicate set of samples which were incubated for the same time in the presence of 10 μM GM6001 (to inhibit MMP activity) from the total fluorescence value of the same sample.

6.2.7 Western blot

MMP-2, TIMP-4, troponin I and MLC1 content in cell lysates were evaluated by Western blot. 2.5 μg protein from each cell lysate was separated using 15% SDS-PAGE[27] and transferred to a polyvinylidene difluoride membrane (PVDF, Bio-Rad). TIMP-4 was recognized using a rabbit anti-human polyclonal TIMP-4 antibody (Chemicon, AB816). Troponin I and MLC1 were identified using a monoclonal anti-human troponin I antibody (Spectral Diagnosis Inc, Clone 8I-7) and a monoclonal anti-human MLC1 antibody (Accurate Chemical and Scientific Corporation), respectively. For MMP-2 detection, 5.0 μg protein from each cell lysate was separated in 8% SDS-PAGE and transferred onto PVDF membranes. MMP-2 was identified using a rabbit polyclonal (Fragment 1) antibody generated from a peptide of the gelatin-binding domain of human MMP-2, as was used in previous studies.[14,15,17] TIMP-4 release into the cell media was also quantified in the same manner by loading 5.0 μg protein/sample. Band densities were measured using GS-800 calibrated densitometer and Quantity One software. Protein loading control was established by both loading the same amount of measured protein and by quantifying the level of β-actin using a rabbit anti-human polyclonal antibody (Santa Cruz, I-19). Membranes were stripped and re-probed by washing with TTBS (3 x for 20 min intervals) and using stripping solution (Pierce).

6.2.8 2-D electrophoresis and mass spectrometry for detection of MMP-mediated MLC1 degradation

Cell lysates for 2-D electrophoresis were prepared as previously described in section 4.2.3. In order to evaluate the feasibility of this technique in cardiac myocyte lysates, we performed a pilot experiment using cell lysates from one trial experiment from each of the treatment groups. Briefly, 10.6 μg of cell lysate protein was applied to 11 cm immobilized pH gradient strips (IPG, BioRad), and equilibrated for 16-18 h at 20°C in rehydration buffer. The 11 cm IPG strips have a linear pH gradient of 5-8. For isoelectrofocussing, the BioRad Protean isoelectrofocussing cell was used as in section 4.2.3.[28] Second dimensional electrophoresis was carried out using Criterion precast gradient gels, 8-16% acrylamide (BioRad). To minimize variations in resolving proteins during the second dimension run, the gels were run simultaneously using a Criterion Dodeca Cell (BioRad). After separation, all the gels were stained using silver staining (BioRad) in the same bath for protein detection. Based on the results of a successful pilot experiment, we decided to run the actual experiment with a n=3 separate myocyte isolations/group based on the limitation that we could not run more than twelve 2-D gels simultaneously (3 gels per group: Control, dec. ONOO$^-$, ONOO$^-$, ONOO$^-$ + doxycycline to give a total of 12 gels). These three analyses were added to the results of the pilot experiment in order to achieve a total of n=4 for spot density analysis. This type of analysis was valid because the spot density for MLC1 was compared to an internal control (reference spot) for each gel. Inter-gel reproducibility of spot intensity was confirmed as previously described in section 4.2.3.

Developed gels were scanned and the quantitative analysis for spot intensity were performed based on the parameters explained in section 4.2.3. MLC1 was identified based on its location in terms of isoelectric point and molecular weight. The degradation products of MLC1 as observed in Chapter 4 were not seen here because of the limited protein amount. The identity of the MLC1 spot was confirmed by mass spectrometry (data not shown). The density of MLC1 was evaluated in all the experimental groups.

6.2.9 Dityrosine formation by ONOO$^-$

An experiment was performed to examine the possibility that doxycycline may act as a direct scavenger of ONOO$^-$. The effect of doxycycline in dityrosine formation from L-tyrosine by ONOO$^-$ was evaluated according to the procedure described by Ferdinandy and Schulz with slight modifications.[29] Briefly, fresh Krebs-Henseleit buffer containing 100 μM Ca^{2+} was mixed with 300 μM L-tyrosine and bubbled with a mixture 95 % O$_2$: 5 % CO$_2$ at 37°C (pH 7.4). Doxycycline was added to 2 mL Eppendorf tubes at different concentrations (3, 10, 30, and 100 μM) in a total volume of 100 μL. An aliquot of the buffer containing L-tyrosine (300 μM, 1700 μL) was added to each tube. 300 μM ONOO$^-$ was quickly added to the tube and it was closed. The tubes were vortexed for 15 s and incubated for 10 min at 37°C. The reaction was stopped by placing the tubes on ice. The entire volume was transferred to 4 mL acrylic transparent cuvettes (Elkay® "Ultra-vu", Shrewsbury, MA) for fluorometric evaluation. To test dityrosine formation, the samples were excited at 320 nm wavelength and scanned between 360 nm and 500 nm wavelengths (scan time 64 s, 0.95 nm/s) in a SLM-Aminco MC 200 monochromator spectrofluorometer (Rochester, NY). In order to determine whether doxycycline may have quenched the fluorescent signal from dityrosine, $ONOO^-$ was added first to the Krebs-Henseleit buffer containing L-tyrosine, incubated for 10 min at 37°C and 100 μM doxycycline (final concentration) was then added. In addition, to determine whether doxycycline fluoresces at the same wavelengths as dityrosine, Krebs-Henseleit buffer containing L-tyrosine was added to doxycycline at the highest concentration and excited at the same wavelength and emission was scanned between 360 and 620 nm.

6.2.10 Statistical analysis

Data are expressed as means ± SE. t-test, one-way ANOVA followed by post-hoc analysis using Tukey's multiple comparison test and the Wilcoxon signed rank test were used for statistical analysis, as appropriate. $p< 0.05$ was the criterion for statistical difference. The n number refers to the number of individual cardiac myocytes isolations.

6.3 RESULTS

6.3.1 Effects of $ONOO^-$ in viability and morphology of cardiac myocytes

The effects of $ONOO^-$ on the viability of cardiac myocytes was determined using trypan blue staining. Viability of control cells declined significantly in comparison to cells which were immediately evaluated after their isolation "Baseline", (Figure 6.2). Doxycycline (100 μM) increased cell viability in comparison to cells treated with ONOO$^-$ alone (43 ± 1 vs. 28 ± 2%, n= 6, $p < 0.05$ respectively). Cells treated with ONOO$^-$ showed a significant 25% decrease in viability compared to control. Dec ONOO$^-$ also caused a small 6% yet significant loss in viability.

6.3.2 ONOO$^-$ induces release/activation of MMP-2 and decreases TIMP-4 release from cardiac myocytes

Cardiac myocytes exposed to ONOO$^-$ had a significant increase in release/activity of 62 kDa MMP-2 in the incubation media as measured by gelatin zymography in comparison to control (Figure 6.3A). 62 kDa MMP-2 activity in doxycycline treated cells did not change against either control or ONOO$^-$. We were not able to evaluate protein level of MMP-2 in the media by Western blot due to the low protein level which is below the detection limit (data not shown). TIMP-4 protein was however detectable by Western blot in the media. Both ~26 and ~46 kDa forms were observed, the former representing a potential post-translational modification of TIMP-4[30] and the latter a potential dimer of the protein. ONOO$^-$ treated cells, in the presence or absence of doxycycline, had less ~26 kDa TIMP-4 in the media than control, as did media from dec. ONOO$^-$ treated cells. The level of ~46 kDa TIMP-4 was unchanged across the treatment groups (Figure 6.3B).

6.3.3 Effects of $ONOO^-$ on MMPs activity determined using OmniMMP substrate

MMPs activity in cell lysates was also determined by a kinetic enzyme assay using OmniMMP substrate. To determine the proportion of the fluorescent signal which was due to MMPs activity, a representative amount of cell lysate (3 μg) was incubated with the OmniMMP substrate in presence of increasing concentrations of the broad spectrum MMPs inhibitor, GM6001. A concentration-dependent partial reduction of substrate metabolism was observed with increasing concentrations of GM6001 with an EC_{50} value of 70.8 nM and a maximal effect observed at 10 μM (Figure 6.4A).

The metabolism of the OmniMMP substrate was assessed in lysates prepared from the various treatment groups. We observed no significant differences in total OmniMMP proteolysis between the groups. After treatment with 10 μM GM6001 to assess net MMPs-related activity, it was observed that ~30% substrate metabolism can be attributed to activity of MMPs (Figure 6.4B). Net MMPs related activity was similar among all experimental groups (Figure 6.4C).

6.3.4 $ONOO^-$ reduces MMP-2 but not TIMP-4 levels in cardiac myocytes

Western blot was performed in order to see changes in both MMP-2 and TIMP-4 levels in lysates from cardiac myocytes. There was a reduction in the level of 62 kDa MMP-2 in cells treated with $ONOO^-$ vs. Control (Figure 6.5A). Both dec. $ONOO^-$ and doxycycline pre-treatment did not change MMP-2 levels in comparison with control.

The level of TIMP-4, whether 29 or 46 kDa, was unchanged in cell lysates from all treatment groups (Figure 6.5B).

6.3.5 Degradation of sarcomeric proteins

We evaluated whether $ONOO^-$ treatment of myocytes increased the degradation of sarcomeric proteins potentially by a MMP-dependent mechanism. There were no differences in the protein levels for either troponin I or its 20 kDa degradation product in the cell lysates (Figure 6.6A). Levels of MLC1 were also evaluated by immunoblot and no differences were observed among the experimental groups (Figure 6.6B). Using the technology as in Chapter 4 the measurement of MLC1 and its degradation products was also attempted using 2-D electrophoresis. The levels of native MLC1 showed no difference among the different treatment groups (Figure 6.7 and 6.8). However, it appeared to be lower in $ONOO^-$ treated cells (44 ± 6 % of control) but this did not reach statistical significance ($p= 0.06$ vs. control). Due to the low levels of MLC1 degradation products, their quantification was not possible.

6.3.6 Doxycycline partially scavenges $ONOO^-$

In order to determine whether the beneficial effects of doxycycline may be mediated in part through a direct scavenging action of $ONOO^-$, the effect of doxycycline on dityrosine formation from the reaction of L-tyrosine with 300 μM $ONOO^-$ in Krebs-Henseleit buffer was evaluated by spectrofluorometry. The reaction of $ONOO^-$ and L-tyrosine resulted in the formation of dityrosine as evidenced by the characteristic peak of fluorescence at 410 nm. Doxycycline caused a concentration-dependent reduction in dityrosine formation to 29 ± 3% as seen with 100 μM doxycycline ($n= 3$, $p< 0.05$ vs. 300 μM $ONOO^-$ alone, Figure 6.9). This effect was not mediated by the quenching of dityrosine fluorescence by doxycycline (data not shown). In addition, doxycycline alone has a weak fluorescence in the tested λ_{em} ranges. However, the peak of fluorescence by doxycycline was observed around 500 nm in comparison to 400-410 nm observed for dityrosine (Figure 6.9).

6.4 DISCUSSION

In this study we found that doxycycline protects adult cardiac myocytes from $ONOO^-$-induced injury. This was observed as an increased viability (by trypan blue exclusion assay) in doxycycline treated myocytes exposed to a single bolus of $ONOO^-$. In addition, we observed that $ONOO^-$ induces the release of MMP-2 from the cardiac myocyte into the media which is accompanied by a decreased level of MMP-2 in the cardiac lysate. However treatment with doxycycline, although showing a trend to normalize MMP-2 to control levels, did not significantly change either the release or the intracellular level of MMP-2.

In this study, we observed that there was a significant reduction in the release of TIMP-4 into the media in all the treatment groups in comparison to control group. One possible explanation of this result is that $ONOO^-$ treatment may have chemically modified the TIMP-4 such that the protein was less avidly recognized by the antibody. In addition, we observed that the molecular weight of the TIMP-4 released into the media was ~26 kDa, which is closed to a ~25 kDa TIMP-4 described by Schulze *et al* which they founding purified thin myofilaments prepared from aerobic rat hearts.[16] This increase in TIMP-4 molecular weight could correspond to the unprocessed 29 amino acid leader sequence found in TIMP-4 from humans.[31] In contrast TIMP-4 of ~29 kDa was observed in the cell lysates and its level was not changed between experimental groups.

This 29 kDa form of TIMP-4 may correspond to the glycosylated form of this protein which is also recognized by the antibody used in this study. We also observed a ~46 kDa band which was consistently found in immunoblots of either media or lysate samples. This could correspond to a putative dimer of TIMP-4 which was observed in the Western blot of TIMP-4 in a recent study performed with mice cardiac fibroblasts however the authors did not mention the identity of the ~46 kDa band.[32] However, to our knowledge the presence of TIMP dimmers was only confirmed for a mutated TIMP-3 found in the retinas of patients with Sorby's fundus dystrophy.[33] We did not test TIMP-4 activity by reverse zymography nor did we confirm whether the 46 kDa TIMP-4 band disappeared after pre-absorbing the TIMP-4 antibody with the antigen in order to rule out the possibility of non-specific binding.

We observed that relative levels of TIMP-4 found in the media were relatively higher than the levels of MMP-2 as seen by immunoblot analysis. Taking into consideration that TIMPs inhibit to MMPs by forming 1:1 complexes with them, it is possible that the overall MMPs activity is increased inside the myocyte as a result of $ONOO^-$ treatment. One way to show this would be by *in situ* zymography which should be done in a future study.

The results of this study did not show that the activation of MMPs by a relatively high concentration of $ONOO^-$ given outside the cell leads to degradation of sarcomeric proteins such as troponin I or MLC1 as we have previously shown in isolated rat hearts subjected to I/R.[15,17] However, we observed that troponin I is already degraded in all cell lysates (including control) and that even though we could not see a difference in levels of MLC1 by immunoblot, we almost saw a difference in MLC1 levels by 2-D electrophoresis. The lack of correlation between this study and studies of I/R injury in the intact heart may be due to differences in the experimental model used (organ vs. isolated cells) as well as potential difference in the concentration of $ONOO^-$ generated during I/R inside the myocyte compared with the administration of a single bolus of $ONOO^-$ outside of the cells. Furthermore, we cannot rule out the possibility that in this particular model MMPs can degrade proteins other than troponin I or MLC1. In addition, we were not able to see MLC1 degradation products possibly as a consequence of the limited levels of protein which were available to load in the gels (10 μg). This is despite the fact that we used silver staining to increase our detection limit in 2-D electrophoresis. Further studies will be necessary to address these aspects.

Continuous infusion of $ONOO^-$ leads to contractile dysfunction in both neonatal[25] and adult rat cardiac myocytes (Chapter 5), as well as in intact isolated rat hearts.[24,34] However, only Chapter 5 analyzed the potential benefit of MMP inhibitors in this model from a functional standpoint. Although, the previous study (Chapter 5) and this one used 300 μM $ONOO^-$, the fact that $ONOO^-$ was infused as a single bolus here and in Chapter 5 was applied as a continuous infusion over a period $\geq$ 10 min may result in differences in terms of how MMPs were activated inside the cell and their effect on potential protein targets.

$ONOO^-$ can cause cell dysfunction through different mechanisms including the post-translational modification of proteins that lead to activation or inactivation of enzyme activity, depending on the concentration. Previously, Lokuta *et al*[35] reported that a bolus of 300 μM $ONOO^-$ inactivated SERCA ATPase pump activity in isolated sarcoplasmic reticulum vesicles from porcine hearts. In addition, vascular SERCA

ATPase pump is activated by low (10-50 μM) and inactivated by high $ONOO^-$ concentrations (100-1000 μM).[36] $ONOO^-$ can cause activation/inactivation of MMPs by post-translational modification such as S-glutathiolation and nitration.[7,37] In addition, $ONOO^-$ inactivates TIMPs thus enhancing the MMP-mediated proteolytical activity inside the cell.[38,39] Therefore, it is plausible in our present study that the TIMP-4 inhibitory effect on MMPs could have been affected by $ONOO^-$ despite the fact that there were no differences in protein level of TIMP-4.

Some authors claim that doxycycline may work as a ROS scavenger.[40] Our results concerning the decrease of dityrosine formation by doxycycline (as a product of the reaction between $ONOO^-$ and L-tyrosine) clearly supports this statement at least in higher concentrations and in the simple non-biological system which was used to test this possibility. Therefore, it can be concluded that the protection observed with doxycycline may in part be related to the direct scavenging effect of $ONOO^-$ along with decreasing proteolysis of unknown protein targets inside the cell.

The isolation procedure to obtain cardiac myocytes causes stress to cells and this may already deplete MMP-2 in myocytes or whether by changes in cell permeability, oxidative stress or even proteolytic cascades stimulated by the use of bacterial collagenase/protease which are necessary to isolate the cells. In addition, because of technical limitations based on the number of cells obtained per isolate and the number of experimental groups we tested only one MMPs inhibitor and we could not compare the results with another such inhibitor with a different chemical structure.

In animal models, doxycycline has been shown to protect from injury caused by acute I/R injury, infarction, sepsis, and stroke by MMP-dependent and independent mechanisms.[13-15,41-44] Recently, it was shown that sub-antimicrobial doses of doxycycline after 6 months of treatment decrease inflammatory markers such as C-reactive protein and interleukin-6 in patients with a previous coronary episode.[45] Therefore, the use of doxycycline or tetracycline related drugs at subantimicrobial doses holds promise in the treatment of the complications of cardiovascular disease.

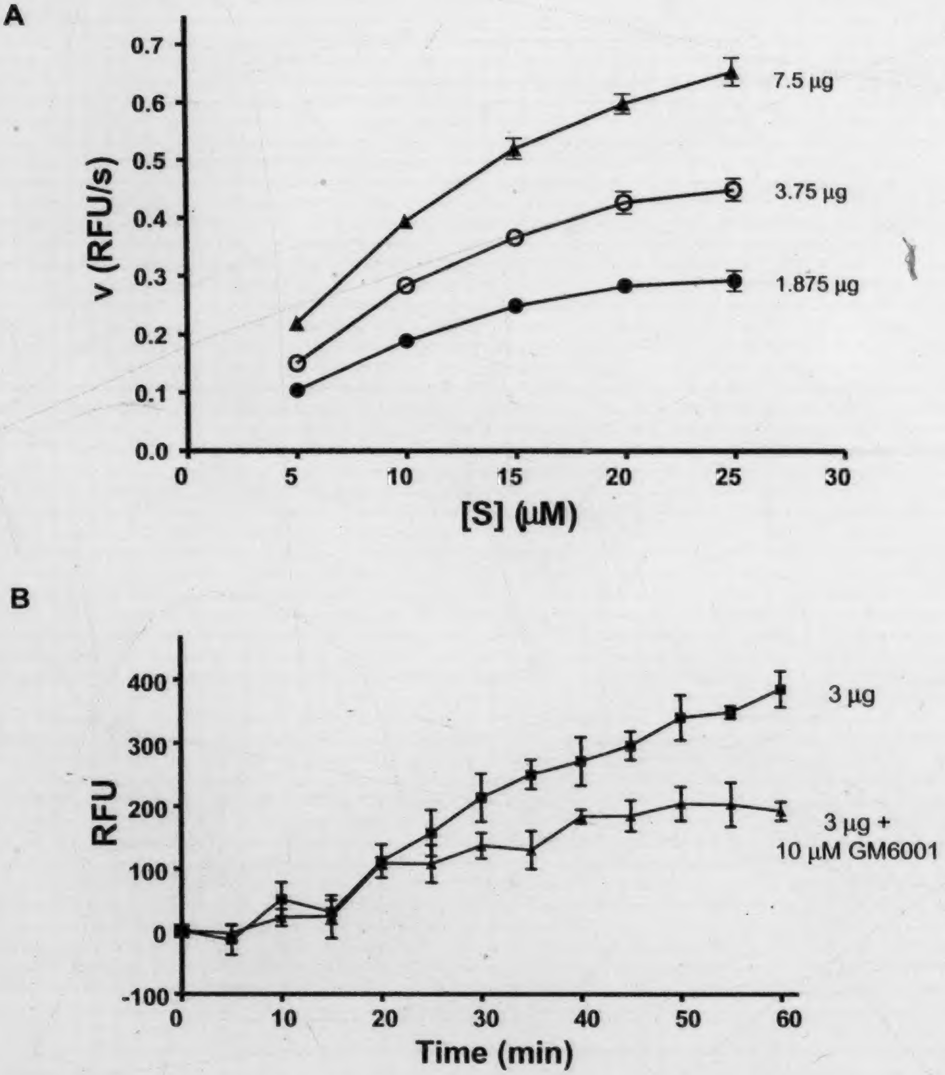

Figure 6.1 Calibration curve for OmniMMP substrate concentration and cell lysates amount for MMPs activity measurement. (A) Fluorescence rate caused by OmniMMP substrate metabolism was measured at increasing amounts of a representative cell lysate performed in triplicate. (B) Time-dependant change in fluorescent signal generated by the metabolism of OmniMMP substrate (15 μM) by a representative amount of cell lysate in the presence or absence of the MMPs inhibitor, GM6001. *(Experiments done with help from A. Holt).*

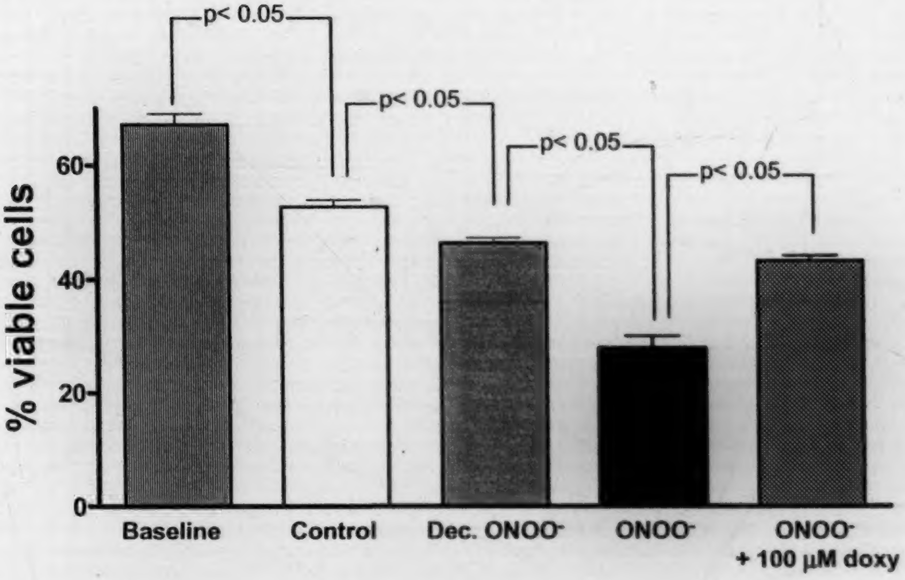

Figure 6.2 Effect of doxycycline on cell viability in ONOO$^-$ exposed myocytes. Bars denote the mean viability as determined by trypan blue exclusion for the different experimental groups from 6 independent myocyte isolations.

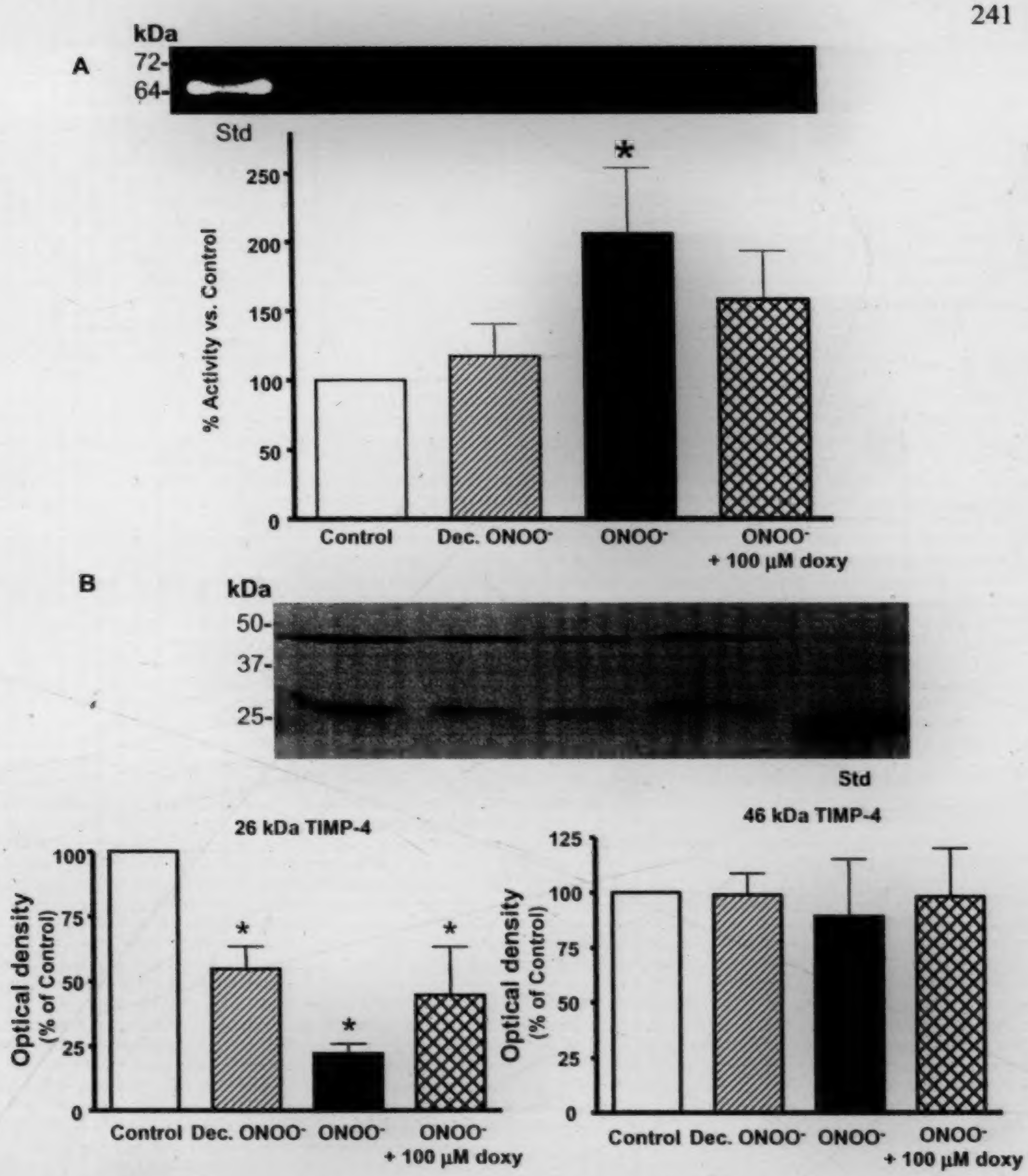

Figure 6.3 Effects of $ONOO^-$ on MMP-2 and TIMP-4 release into the media. (A) Representative zymogram of incubation media from myocytes subjected to dec. $ONOO^-$, or $ONOO^-$ ± doxycycline (doxy). Lower panel depicts summary data of the densitometric analysis of 62 kDa MMP-2 activity. Note that the HT1080 cell supernatant used as a standard (Std) is human MMP-2 which is comprised of both 72 kDa and 64 kDa forms, the latter which is slightly higher in molecular weight than the 62 kDa rat MMP-2. (B) Representative immunoblot of TIMP-4 from the media whowing the presence of both ~26 kDa monomer and ~46 kDa TIMP-4. Recombinant human TIMP-4 was used as standard (Std). Lower panels show the densitometric analysis for both ~26 and 46 kDa TIMP-4 bands. $*p < 0.05$ vs. Control, n=6 independent myocyte isolation experiments.

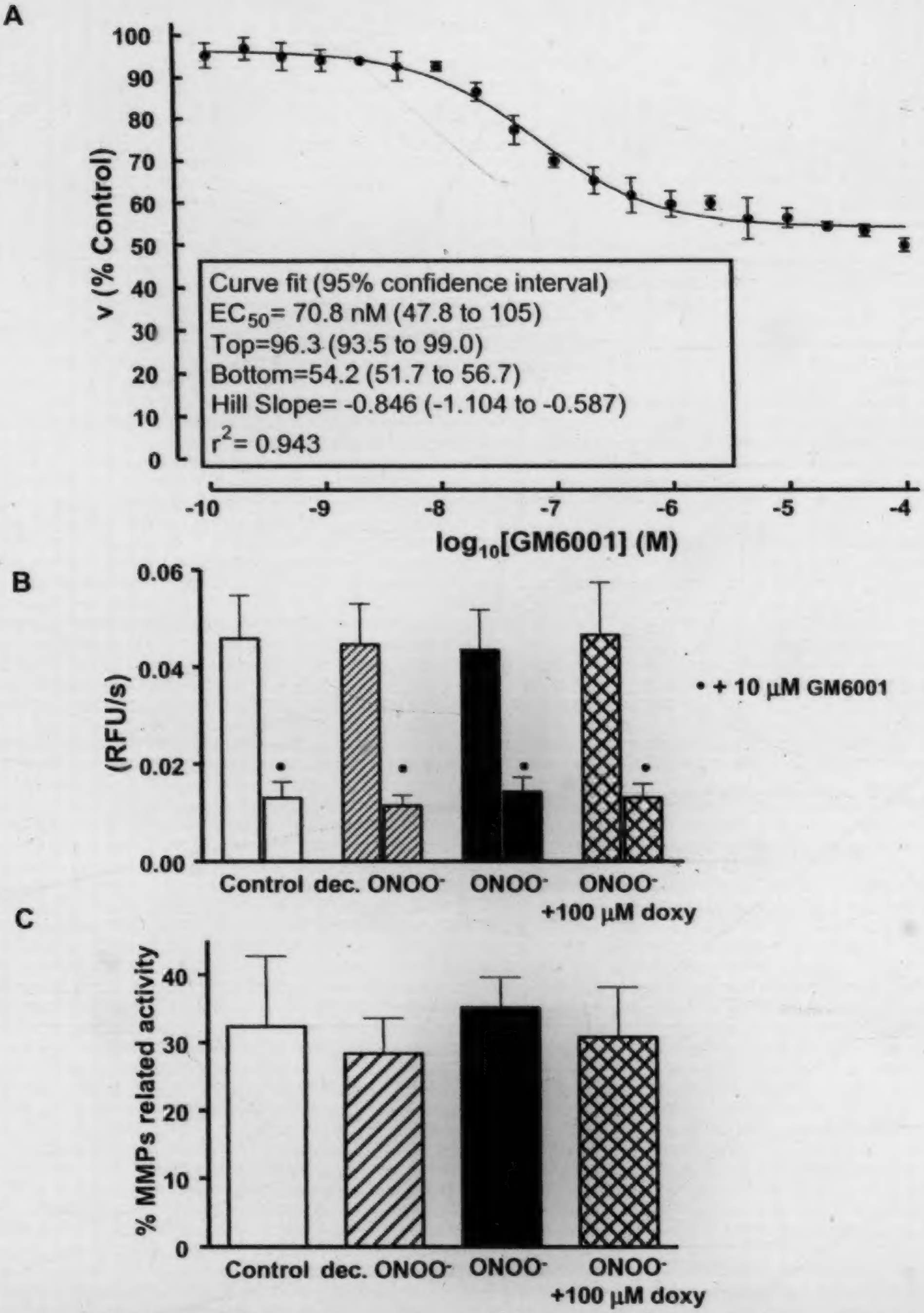

Figure 6.4 Effect of MMPs on OmniMMP substrate metabolism during ONOO⁻ exposure in cell lysates. (A) Concentration response curve of the effects of GM6001 on OmniMMP metabolism by a representative cell lysates, performed in triplicate. (B) Effect of ONOO⁻ and doxycycline on substrate metabolism in presence or absence of GM6001. (C) Effect of ONOO⁻ and doxycycline treatment on net MMPs related activity in myocyte lysates, n=6 independent cell isolations. All experiments were performed in triplicate. *(Experiments done with help from A. Holt).*

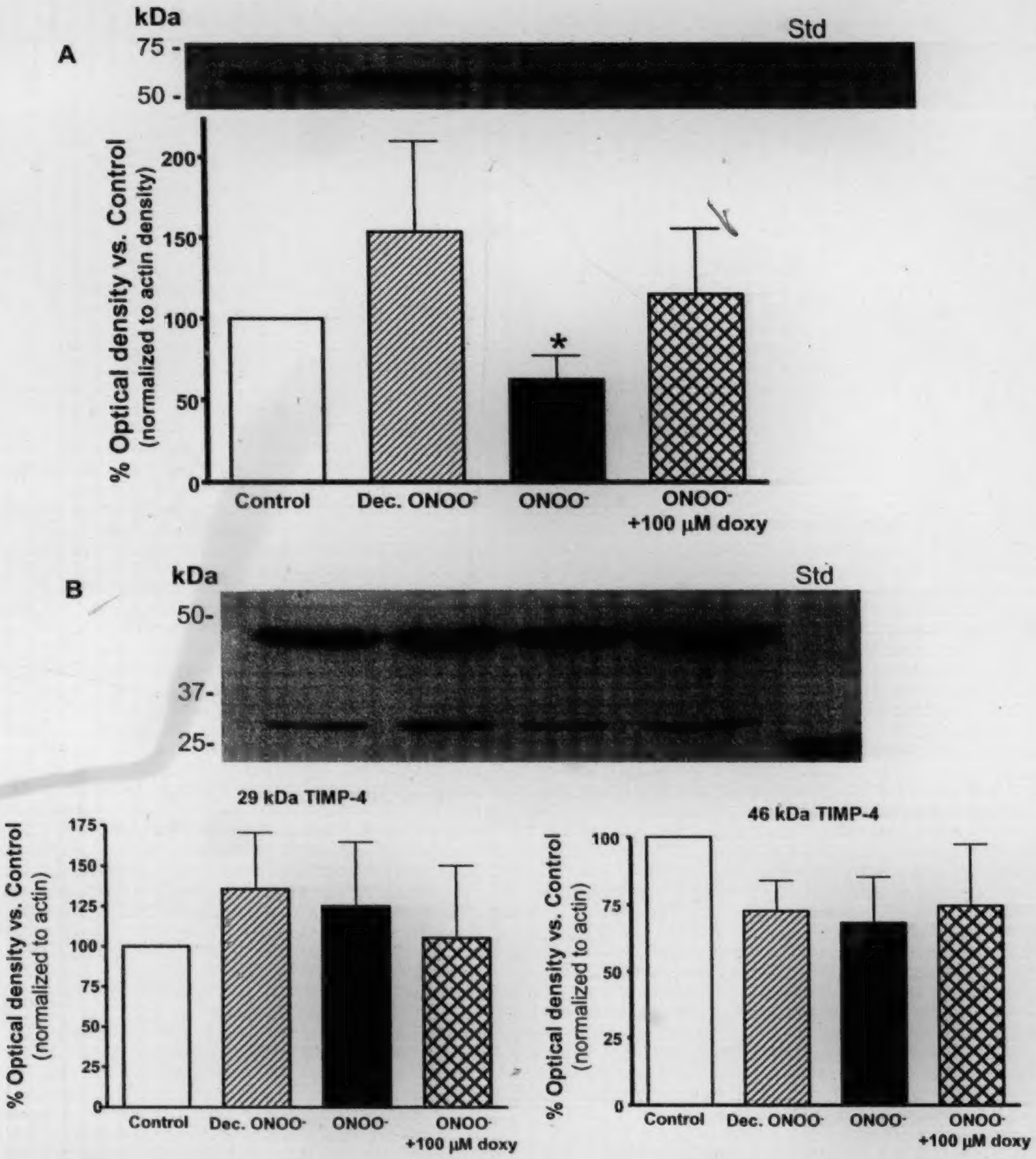

Figure 6.5 Effects of $ONOO^-$ on MMP-2 and TIMP-4 levels in cardiac myocyte lysates. (A) Upper panel, representative immunoblot of 62 kDa MMP-2 in lysates from myocytes subjected to dec. $ONOO^-$, or $ONOO^-$ ± doxycycline. Lower panel depicts summary data of the densitometric analysis of 62 kDa MMP-2 levels from 5 independent isolations. Human recombinant 62 kDa MMP-2 was used as a standard (Std). (B) Representative immunoblot of TIMP-4. The lower panels show the densitometric analysis for both the 29 and 46 kDa TIMP-4 bands, n=6 independent isolations. Human recombinant TIMP-4 was used as a standard (Std).

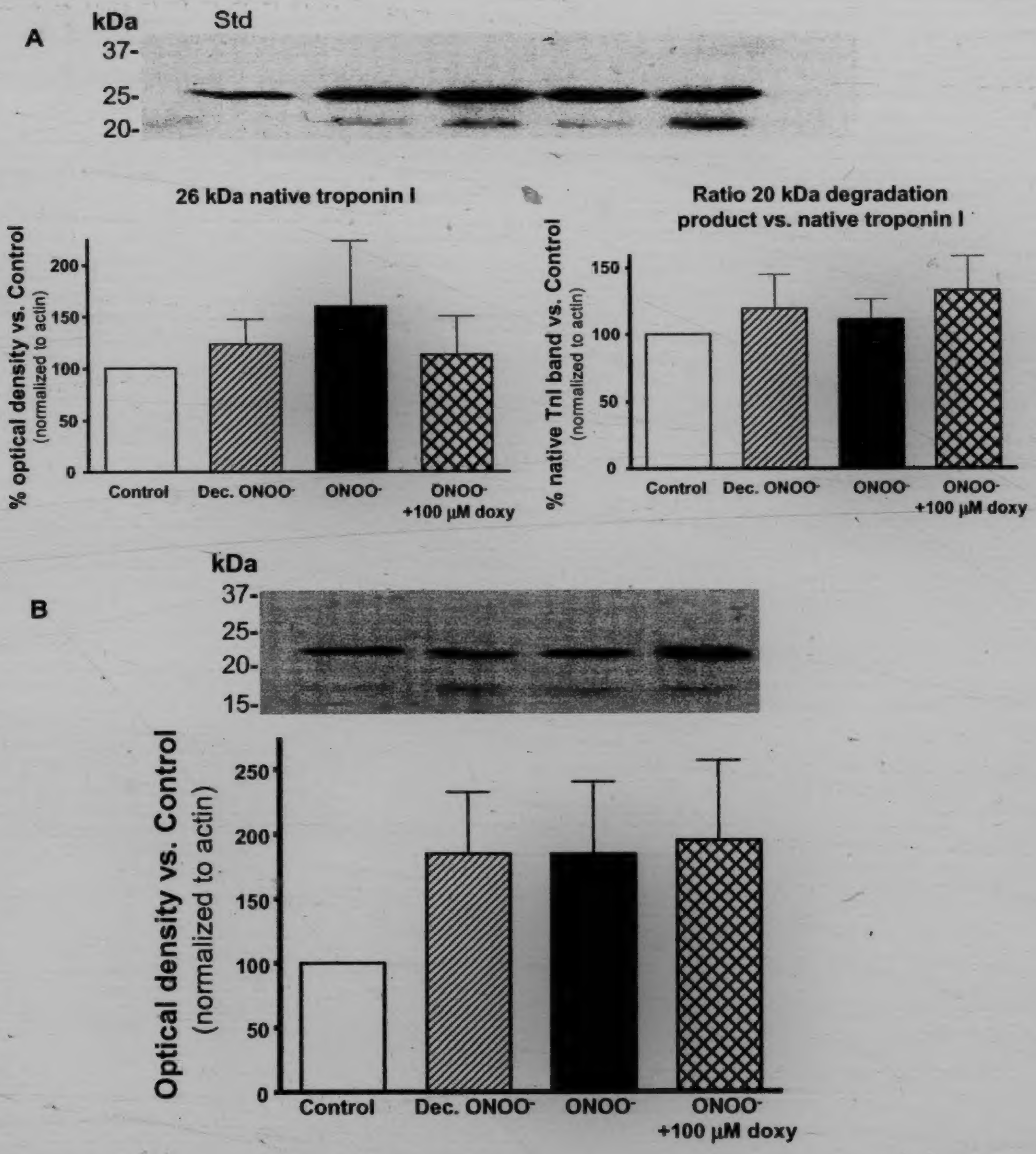

Figure 6.6 Effects of ONOO⁻ on levels of troponin I and myosin light chain-1 (MLC1) in cardiac myocyte lysates. (A) Upper panel, representative immunoblot for troponin I and its 20 kDa degradation product. Lower panel depicts the densitometric analysis for the native troponin I band (left) and the 20 kDa degradation product (right) expressed as a percentage of the native troponin I band from the same treatment groups. Human recombinant 26 kDa troponin I was used as a standard (Std). (B) Representative immunoblot of MLC1. Lower panels show the densitometric analysis for the treatment groups. n= 6 independent isolations for both (A) and (B).

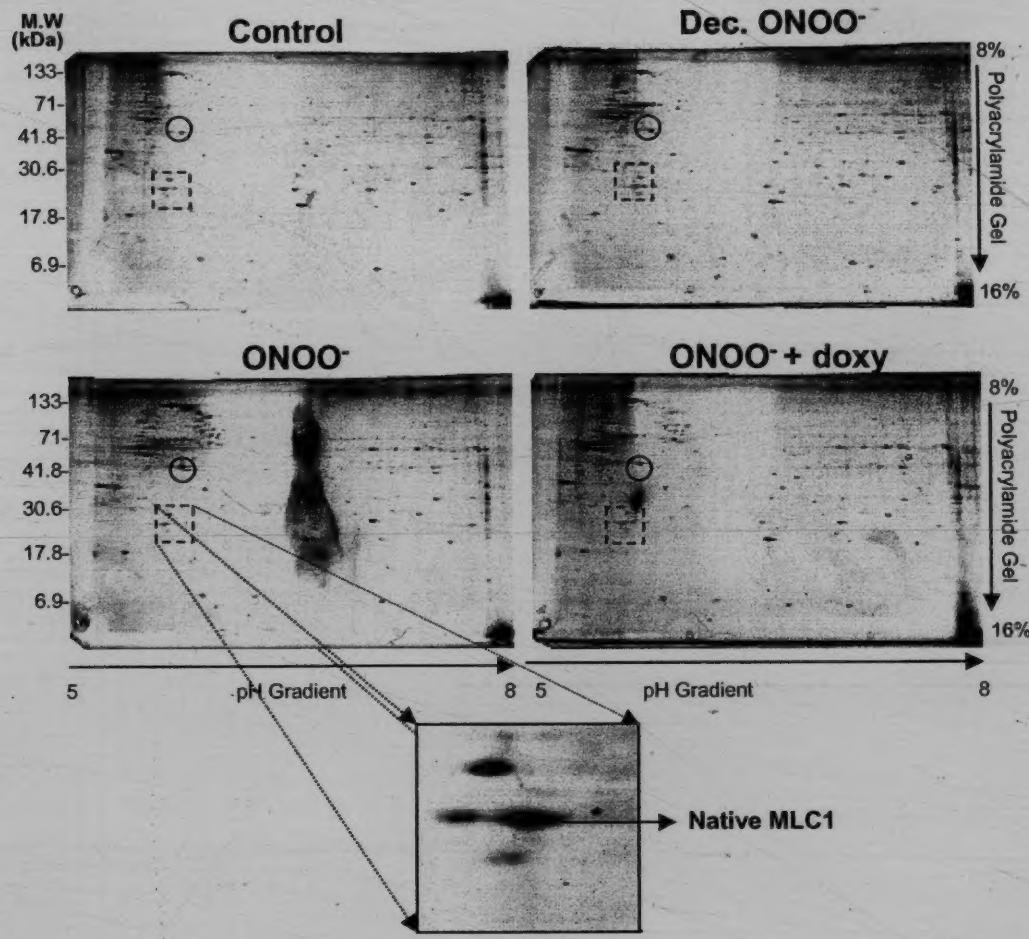
M.W (kDa)
Control
Dec. ONOO⁻
133-
71-
41.8-
30.6-
17.8-
6.9-
8%
Polyacrylamide Gel
16%
ONOO⁻
ONOO⁻ + doxy
5
pH Gradient
8
Native MLC1

Figure 6.7 Representative 2-D electrophoresis of cardiac myocytes lysates from Control, dec. ONOO$^-$, ONOO$^-$ and ONOO$^-$ + doxycycline groups using a narrow range pH strip (5-8) and 8-16% polyacrylamide gradient gel. 10 μg total proteins were loaded per gel. A representative silver stained gel from each group is shown. The square indicates the region of the gel where MLC1 is localized. The spot on the circle is the reference spot used for comparison purposes in the quantitative analysis. Representative enlargement of the gel showing the marked region is shown. *(Experiments done with help from G. Sawicki).*

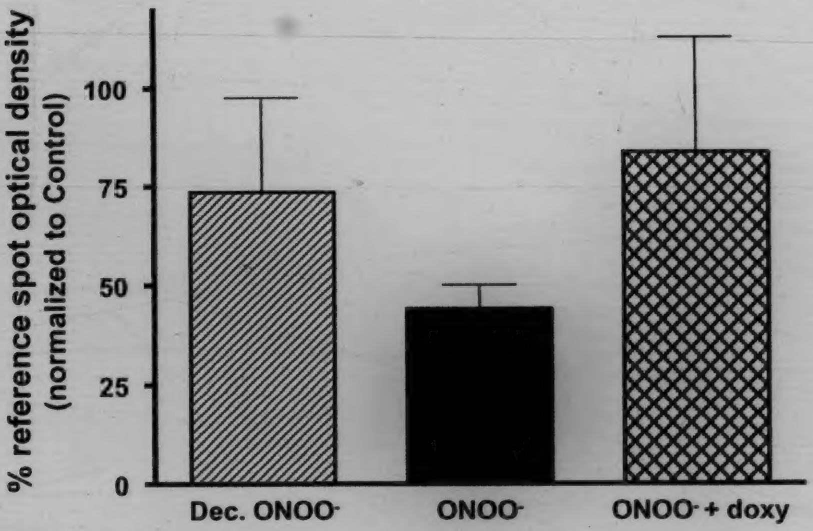

Figure 6.8 Summary data of densitometric analysis for native MLC1 from 2-D electrophoresis. Bar graph shows the optical density for the native MLC1 protein spot in 2-D electrophoresis, n=4 independent cell isolations.

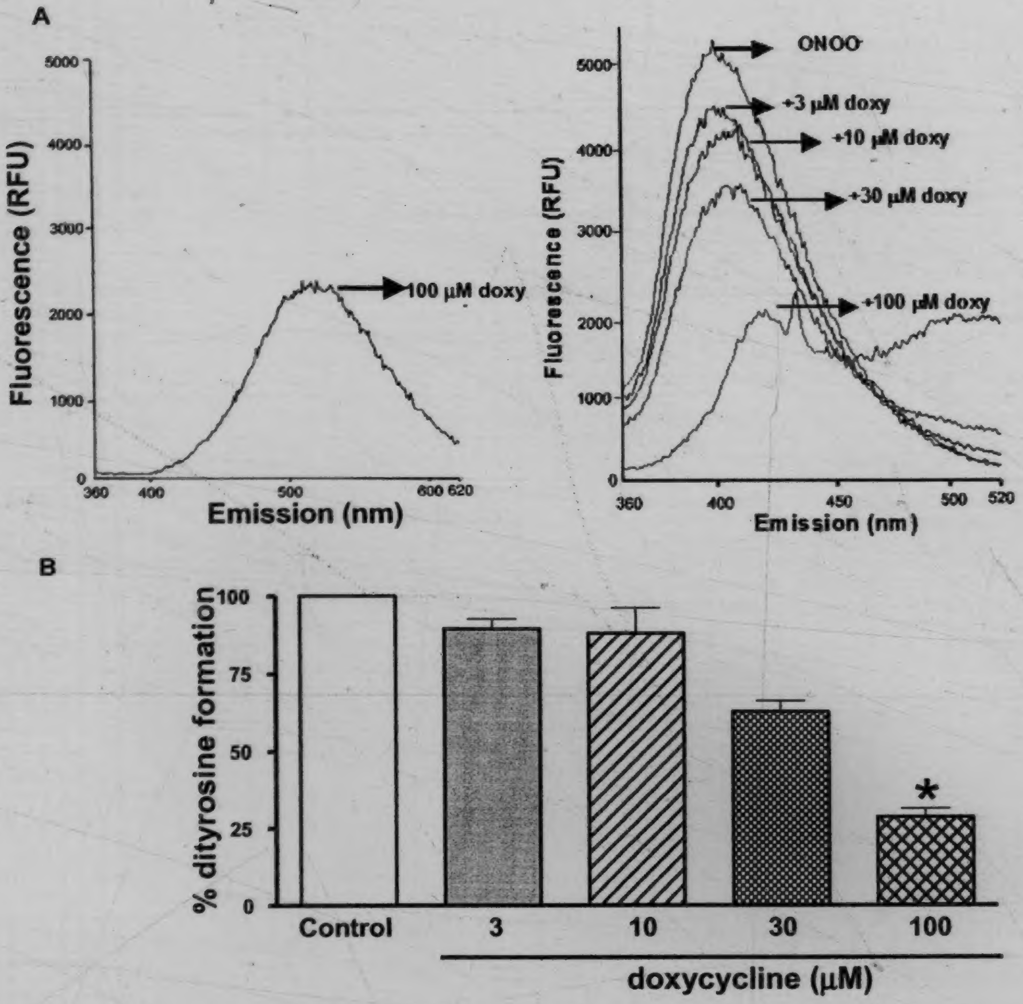

Figure 6.9 Effects of doxycycline on dityrosine formation after reaction of L-tyrosine with 300 μM ONOO$^-$. (A) Representative fluorescent spectra at λ_{ex}= 320 nm for doxycycline alone (left) and dityrosine levels formed by the reaction of ONOO$^-$ and L-tyrosine in the presence of increasing concentrations of doxycycline (right). (B) Quantitative analysis of dityrosine formation. * $p < 0.05$ vs. Control, n=3 experiments.

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CHAPTER 7

CONCLUSIONS, LIMITATIONS AND FUTURE DIRECTIONS

7.1 CONCLUSIONS

In this thesis, I explored the role of oxidative stress in the activation of two distinct proteins, AMPK and MMP-2, both considered to be important in cardiac physiology and pathology. I reported the novel observation that in the isolated working rat heart AMPK is rapidly activated upon exogenous oxidative stress with H_2O_2. This could be related to cardiac injury because both AMPK activation and contractile dysfunction caused by H_2O_2 are prevented by the antioxidant pyruvate. In addition, using the same model, I showed that MMP-2 is activated and released into the coronary effluent upon administration of H_2O_2. However, in this particular model inhibition of MMPs did not significantly protect against this specific injury. In addition, direct infusion of $ONOO^-$ caused contractile dysfunction in isolated adult rat cardiac myocytes which was prevented by pharmacological inhibition of MMPs. Consistent with this, I also showed that doxycycline improves the viability of cardiac myocytes challenged with a bolus of $ONOO^-$ through inhibition of MMPs and in part by scavenging $ONOO^-$. Using I/R injury as a model of endogenous generation of oxidative stress, I demonstrated that MMP-2, which is activated during this injury proteolyzes MLC1, thus describing a new intracellular target for MMP-2 in the sarcomere.

When oxidative stress outstrips the endogenous antioxidant cell defences modification of multiple proteins targets occurs, leading to cell damage and cell death. In my first study, I tested the hypothesis that H_2O_2 can activate AMPK. AMPK was previously shown to be activated upon stress conditions that decrease ATP stores such as I/R and hypertrophy. (discussed in Chapter 1). I showed for the first time in the isolated working rat heart that AMPK activation parallels the cardiac mechanical dysfunction caused by 5 min exposure to H_2O_2. Whether this is a protective or causative mechanism of injury remains to be addressed as an AMPK inhibitor was not able to prevent injury by H_2O_2.

In my second study using isolated working rat hearts exposed to H_2O_2 for a longer period of 60 min I observed that there is an increase in MMP-2 release and activity in the coronary effluent which peaked at the time of maximal cardiac depression (5 min). Antioxidants such as pyruvate prevented not only the cardiac depression but also the release of MMP-2 in the coronary effluent. However, MMPs inhibition did not rescue the decline in cardiac function and this could be related to the widely known effects of H_2O_2 on lipids, other proteins and ionic homeostasis which may have overwhelmed any beneficial effects of inhibiting MMPs activation.

My third study explored the possibility that MMP-2 activation inside the cardiac myocyte during I/R can have sarcomeric targets other than troponin I. Using a novel pharmaco-proteomics approach I found a novel substrate for MMP-2 in this model which is MLC1. In addition, by using mass spectrometry and bioinformatics tools, I was able to determine the exact cleavage site where MMP-2 proteolyzes MLC1 to be between tyrosine 189 and glutamine 190 at the C-terminal domain. These very exciting results may shed light on the role of MMP-2 in the pathophysiology of I/R, and warrant further clinical investigation. Moreover, the development and use of the pharmaco-proteomics approach may contribute to establish a basis for new cardiac research projects.

In my fourth and fifth studies, I explored the hypothesis that $ONOO^-$ causes contractile dysfunction via activation of MMPs in isolated adult cardiac myocytes. I found that the contractile dysfunction caused by $ONOO^-$ is attenuated by pharmacological inhibition of MMPs using two distinct MMPs inhibitors. This is the first study showing that the activation of MMPs and the consequences on cardiac contractile function are independent from their action on extracellular matrix proteins, as previously described in remodelling after myocardial infarction as well as in other experimental models such as assessing lung tissue repair using human fetal lung fibroblasts.[1,2] I continued exploring this avenue by testing the hypothesis that doxycycline protects myocytes from $ONOO^-$-mediated damage by inhibiting MMPs and thereby preventing the degradation of troponin I and MLC1. I found in this study that the addition of a single bolus of $ONOO^-$ induced release of MMP-2 into the media and that doxycycline improved myocyte viability after challenge with $ONOO^-$. However, doxycycline treatment showed a trend to normalize MMP-2 levels. MLC1 degradation also trended to be increased in $ONOO^-$ treated cardiac myocytes in comparison to control cells, however, doxycycline did not appear to prevent the degradation of troponin I or MLC1. Perhaps under the exact conditions of this experiment or using different ONOO- concentrations or incubation times the results mentioned above would be more conclusive. Based on evidence that $ONOO^-$ inhibits TIMPs activity,[3,4] it is plausible that in this study an imbalance between TIMPs and MMPs may occur inside the cell leading to an enhanced intracellular proteolytical activity and consequent proteolytic cleavage of other protein targets besides the ones that I expected in this study. For example recently it was found that the cytoskeletal protein α-actinin is susceptible to degradation by MMP-2 (R. Schulz unpublished observations) and therefore should be tested for in future experiments. In a non-biological system I found that doxycycline at the concentration used in this study partially scavenges $ONOO^-$. Thus, the protective effect of doxycycline in $ONOO^-$-challenged cardiac myocytes is related to its inhibitory effect on MMPs and in part by a direct scavenging of $ONOO^-$.

7.2 LIMITATIONS

7.2.1 General limitations

There are several limitations that should be acknowledged and considered for future studies in this interesting area of heart research.

One of the techniques used during this thesis was the perfusion of isolated rat hearts or isolated cardiac myocytes with crystalloid buffers which mimic but do not replace perfusion with blood or *in vivo* experimental models as well as the absence of neurohormonal regulation in isolated hearts and isolated cardiac myocytes brings difference to what may be seen in *in vivo* conditions. Therefore, extrapolation of these results to pathological events that occur *in vivo* must be done with caution. In addition, in Chapters 3 and 4 the use of crystalloid buffers in either working or Langendorff perfusion models can cause some edema and this may induce release of MMP-2 into the effluent. MMP-2 activity can be observed in fluid samples from patients with hydrostatic lung edema.[5]

Through this thesis one of the techniques most used is gelatin zymography, a well established assay which determines both the molecular weights and activity of MMP-2 and MMP-9. This technique is highly sensitive and reproducible. However, several limitations can be accounted to this technique: First, gelatin zymography only reveals MMP-2 and MMP-9 activities. No information on any of the other MMPs activities which can be found in the heart were not evaluated in all the chapters of this thesis.

Second, gelatinolytic activity of MMP-2 was not always compared to the levels of MMP-2 by immunoblot. However, it is important to mention at this point that the gelatinolytic activity assessed by this technique does not always correlate with the protein levels.[6,7] Moreover, the non-reducing conditions of the gel activates latent proteins as well as dissociates MMP-TIMP complexes along with separation of the MMP-MMPs inhibitor complex during electrophoresis.[8] Therefore one should supplement assays of MMPs activity by zymography using other techniques such as substrate degradation assays including specific synthetic peptides, degradation of known substrates such as gelatin, collagen and troponin I and other techniques that may be developed in the future to test "net" MMPs activities.

The use of freshly isolated cardiac myocytes has its limitations. The use of bacterial-derived collagenase and protease to dissociate the myocytes from the extracellular matrix may lead to activation, depletion and cleavage of endogenous MMPs and other proteases as well as other cell stresses. This event might be reflected in the absence of 72 kDa MMP-2 in zymography and Western blots discussed in Chapters 5 and 6 as well as by the fact that even immediately after isolation cell viability is only ~70%.

7.2.2 Limitations to Chapter 2

The first major limitation in this study is that the effect of AMPK activation on its downstream targets, such as acetyl-CoA carboxylase (ACC) and endothelial nitric oxide synthase (eNOS), was not explored. In addition, whether the activation of AMPK by H_2O_2 was due to the activation of any of its upstream activators AMPK kinase (AMPKK) or the serine/threonine kinase, LKB-1, was not tested in this study.

A potential limitation of this study is that the perfusion buffer in this model did not contain radiolabelled substrates to evaluate the effects of AMPK activation on fuel metabolic pathways during these experiments. However, this study was not designed to study the metabolic pathways which occur in the heart.

Finally these results are difficult to extrapolate to the clinical setting due to first, this study used a supra-physiological (5 mM) concentration of pyruvate which cannot be successfully administered to patients due to the risk of precipitation or hypernatremia (pyruvate is normally administrated as a sodium salt).[9] However, a recent clinical trial showed that the administration of pyruvate at 10 mM in the cardioplegic solution was not only safe but also beneficial (measured in terms of reduced release into the coronary sinus of troponin I and creatine kinase-MB, improvement of left ventricular function as well as enhanced myocardial oxygenation) in patients subjected to coronary bypass grafting surgery.[10]This clinical study had its own limitations of a small sample size (n=15/group) and that it was not a blinded.

7.2.3 Limitations to Chapter 3

This chapter evaluated the possible role of MMP-2 in cardiac contractile dysfunction induced by H_2O_2. This is the first study which address this aspect using as a experimental model isolated heart perfusions in comparison to cell culture models. However, this study does not show a clear association of MMP-2 activation by H_2O_2 to the magnitude of cardiac dysfunction due to the lack of protection with MMP inhibitors. In addition, I could not determine the concentration and stability of H_2O_2 during the course of the experiment after it was added to the perfusion buffer due to technical limitations. If for example a highly sensitive and specific fluorogenic substrate that is activated in the presence of H_2O_2 would be available, this would help one to follow the stability of this ROS and could overcome some of the uncertainty of the stability of H_2O_2 over the course of the experiment.

Another limitation of this study was that in the series where MMPs inhibitors were evaluated I did not measure the release of MMP-2 into the perfusate which makes a comparison to the series of hearts challenged to H_2O_2 in the presence or absence of pyruvate difficult from this standpoint.

Some possible future studies to determine the consequences of MMP-2 activation in this model could include the use of either MMP-2 knockout or overexpressing mice as well as the potential administration of a neutralizing MMP-2 antibody or stable peptides which selectively inhibit MMP-2 activity that might cross the cell membrane after alterations of cell membrane permeability caused by H_2O_2.

7.2.4 Limitations to Chapter 4

This chapter reveals a new protein target for MMP-2 in the isolated rat heart subjected to I/R injury. However, this study has several limitations. The use of two different broad spectrum inhibitors of MMPs does not allow me to draw conclusions regarding the role of specific MMPs. In addition, these inhibitors function as chelators of the Zn^{2+} atom in the catalytic domain and they may have some possible effects on other Zn^{2+}-dependent enzymes. Recently, the interaction with the structural Zn^{2+} and/or Ca^{2+} atoms of MMP-7 has been described as a novel mechanism for MMPs inhibition by doxycycline.[11]

The proteomic approach used in this study has its own limitations. Samples prepared for these experiments only represent intracellular (cytosolic, mitochondrial, nuclear and from other organelles) proteins without containing membrane-associated proteins. Coomassie blue stained was used to detect proteins in the 2-D gels. This stain has a lower general protein detection limit and thus will reveal only more abundant proteins in comparison to other stains such as silver nitrate. Most of the proteomic analysis was performed in 2-D gels that were run looking for proteins that were in a narrow isoelectric point range (5-8). Thus less abundant proteins potentially affected by MMPs as well as those located at different isoelectric points would not be revealed in this analysis. Moreover, although the gels had a broad range (4-16%) for the second dimension proteins >250 or < 10 kDa would also not detected in this study. A final limitation concerning this approach is that because of technical limitations, only twelve gels can be run simultaneously thus leading to small n numbers of samples when more than two experimental groups are evaluated. It also remains to determine whether the degradation of MLC1 by MMP-2 occurs in the human heart.

7.2.5 Limitations to Chapter 5

The lack of a reliable technique to measure intracellular activation of MMP-2 in real time upon $ONOO^-$ challenge leads to difficulty in identifying which compartment(s) MMP-2 is activated. In addition, there is not a precise method to quantify what is the real concentration of $ONOO^-$ that reaches inside the cell during the performance of the functional studies. Due to the short half-life of $ONOO^-$, as well as its photosensitivity, it is likely that the concentration of $ONOO^-$ that finally reached the cells was less than 300 μM.

Another limitation of this study is that it was not possible to collect cells after the experiment due to technical constraints. Thus downstream molecular and biochemical assays were not feasible such as exploring exactly which MMP-2 (i.e 72 or 62 kDa) is activated and by which post-translational modification.

7.2.6 Limitations to Chapter 6

This studied aimed to find the potential protein target for MMP-2 in cardiac myocytes after $ONOO^-$ administration. Some limitations of this investigation are: firstly, in comparison to Chapter 5, $ONOO^-$ was administrated as a single bolus followed by 5 minute incubation, whereas in Chapter 5 continuous infusion over > 10 min was employed. This could result in discrepancies, for example, in the molecular modifications caused by MMP-2 occurring on the proteins of interest in the two studies. As potential targets for MMP-2 in this kind of injury, I focused on sarcomeric proteins that were shown to be targeted by MMP-2 in I/R or cytokine-induced injury such as troponin I and MLC1.[12-14] My inability to detect clear effects on these two putative targets of MMP-2 activity does not preclude the possibility of biologically relevant changes that were beyond the resolution of the assays employed here. Moreover, I was not looking for other less well characterized protein targets (such as cytoskeletal proteins) in this study that might be affected in important ways by the action of $ONOO^-$ on MMP-2 activity.[15] The results obtained are only related to the application of a single bolus of $ONOO^-$ (300 μM) being added outside the cell for a total of 5 min at room temperature. Future studies exposing cardiac myocytes to ONOO$^-$ at the same as well other concentrations will be required to solve this question.

The pharmaco-proteomics approach used in this study directed specifically to detect changes in the levels of MLC1. This excluded the possible effects of MMPs on other potential protein targets in the cardiac myocyte after oxidative challenge with ONOO$^-$. Although I used silver staining in this study which is more sensitive than Coomassie blue employed in Chapter 4, I could not quantify MLC1 degradation products due to low total cellular protein levels available to load the gels.

Another limitation is related to the inability to accurately quantify specific MMP-2 related activity in cell lysates. In order to partially overcome this limitation, I used the catabolism of the omniMMP substrate as a marker of MMP activity. Despite the fact that this fluorogenic substrate is marketed as an MMP-selective (but not MMP-2 specific) substrate, as shown in Figure 6.4, it is clearly degraded to some extent by proteases unaffected by high concentrations of GM6001. Therefore, I could not address the specific MMP-2 activity inside the cell after treatment with ONOO$^-$ in the presence or absence of doxycycline.

Finally, based on previous evidence suggesting that TIMPs could be inhibited by ONOO$^-$,[3,4] it would be desirable to correlate the protein levels of TIMP-4 with its activity in future studies by reverse zymography as well as possibly understand the post-translational modifications of TIMP-4 which may have occurred and which may have altered its biological properties.

7.3 FUTURE DIRECTIONS

This thesis presents exciting results which open an avenue for future studies in the area of cardiovascular degradomics. However, caution should be taken in how these data are extrapolated to the human population. The finding that in the heart oxidative stress caused by H_2O_2 administration activates AMPK leads us to consider future studies in order to clarify the role of this interesting and multi-functional protein in this kind of injury. In addition, the methodology used for this study may help in the design of future studies in which novel AMPK activators or inhibitors can be tested.

The results of these studies provide compelling evidence of the role of ROS in the activation of MMPs in the heart. In addition, they suggest that the magnitude and pathophysiological consequence of MMPs activation in the heart after oxidative stress challenge is different depending on the ROS used (mild for H_2O_2 and more severe for $ONOO^-$). However, future studies comparing simultaneously the effect of similar concentrations of these two ROS on MMPs activity using the same experimental model may address this question.

This thesis thus enhances the association between ROS and MMP activation, and will inform future studies aimed at determining which post-translational modifications MMP-2 undergoes to become activated in the heart during oxidative stress.

Finding a new intracellular target for MMP-2 during I/R such as MLC1 is very exciting and it provides an insight into the pathophysiological role of MMP-2 in the heart. This finding also opens the opportunity to explore whether the same event occurs in the human heart subjected to I/R injury such as during coronary artery bypass grafting surgery (CABG), pharmacological (using thrombolytics) and interventional (angioplasty ± stent) reperfusion. In addition, it underlines the need to evaluate the possible beneficial role of MMPs inhibition (i.e. doxycycline or chemically modified tetracyclines) in clinical trials of patients who undergo CABG surgery or interventional reperfusion. Moreover, with the clear association between the level of oxidative stress and MMPs activation, it will be interesting to explore if adding proper antioxidants in the cardioplegic solution might bring about beneficial effects such as the reduction of I/R injury.

From the technological standpoint the methods developed and used in this thesis could provide a template on which to develop future studies aimed at determining specific changes in the rat heart proteome caused by MMPs after administration of ROS, and further exploration of the role of these potential modifications in protein function. In addition, the pharmaco-proteomics approach not only can be used to study the effects of MMP inhibitors on MMP-related events but also to test different pharmacological approaches in various areas of cardiac research. Additionally, future studies can be designed using this pharmaco-proteomic approach to study the effect on the proteome of specific subcellular fractions such as mitochondria and nuclei or membrane proteins in the setting of a specific physiological or pathological condition.

Finally, the measurement of functional parameters in isolated cardiac myocyte will contribute to our understanding of the role of intracellular MMPs in the heart during different physiological (such as aging) and pathological (I/R, hypertrophy and heart failure) processes.

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